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Studies on Ribonuclease, Ribonucleic Acid and Protein Synthesis in Healthy and Zinc-Deficient Citrus Leaves

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Introduction

The building-up and breaking-down of proteins, fundamental parts of the metabolism of plants, are easily affected by various nutritional factors, particularly zinc. Thus it has been reported that in zinc-deficient plants amides and amino acids accumulate and a continued proteolysis has been found (Hoagland, 3, Wood and Sibly, 11). The addition of zinc to such plants caused protein synthesis in leaves (Wood and Sibly, 11). There is considerable evidence that ribonucleic acid (RNA) is intimately involved in protein synthesis (Gale, 1, Kessler, 5), and the question now arises whether zinc also affects RNA, or related factors, *e.g.* the RNA degrading enzyme ribonuclease (RNAase) which, *in vitro*, is strongly inhibited by zinc ions (Holden and Pirie, 4). RNAase was, therefore, chosen as the first subject for these studies.

This paper describes some properties of RNAase from healthy citrus leaves, and its relative activity in both healthy and zinc-deficient leaves. In addition some relations between protein and RNA were investigated.

Materials and Methods

Leaves of the spring flush (about 6 to 7 months old) were sampled from 27 years old Shamouti orange trees, budded on Sweet Lime and growing on light sandy loam. Some of these trees showed the characteristic mottling of leaves due to zinc de-

Table 1. *Fresh and dry weights of healthy and zinc-deficient Citrus leaves.*

Leaf matter	Healthy leaves	Zinc-deficient leaves
Fresh weight (mg. per leaf)	1,025	470
Dry weight (% of fresh weight)	33	33
Fresh weight (mg./sq. cm.).....	25	27
Dry weight (mg./sq. cm.).....	8	9

iciency, while on neighboring trees no such visual signs could be detected. As "zinc deficient" only medium to severely mottled leaves of reduced size were collected. For comparison green leaves of normal dimensions were sampled only from those trees which had no mottled leaves (Table 1).

Leaf samples were thoroughly washed, first with a detergent and then 3 times with glass distilled water. RNAase was extracted by blending 20 g. of fresh leaves for 5 min. with tenfold their volume of 0.2 *M* sodium citrate buffer (pH 6.5). Initial phosphorus content of the enzyme solution was determined by the vanadate-molybdate method (Gericke and Kurmis, 2), and it was found to be about 30—40 μ g/ml.

Usually 5 ml. of the crude enzyme solution were incubated for 3 hours with 10 ml. of a 0.5 % solution of commercial RNA (Nutritional Biochemical Corporation, Cleveland, Ohio) containing 8.31 % phosphorus and 14.9 % nitrogen. The RNA substrate was dissolved in 0.025 *M* sodium citrate buffer. After incubation the undigested RNA was precipitated overnight by 5 ml. glacial acetic acid and 100 ml. of 95 % ethanol. Phosphorus was determined in the filtrate after wet digestion. RNAase activity was obtained by subtracting the initial phosphorus content of the extract from the figure obtained after incubation. RNAase activity is expressed as percent hydrolysis of RNA after incubation.

Total and alcohol-insoluble nitrogen were determined by a modified microkjeldahl method (Kessler, 5, Markham, 7). Henceforth the alcohol-insoluble nitrogen fraction is referred to as "protein" nitrogen.

RNA was analysed as described previously (Kessler, 5). Zinc was determined by the dithizone method (Sandell, 10).

Results

RNAase activity has been demonstrated in various plant species (Holden and Pirie, 4). In this work RNAase activity was also found to occur in healthy citrus leaves. There is a certain variability in the activity of RNAase of leaf samples, collected on various occasions. The average activity is 41 ± 2 % hydrolysis after 3 hours incubation at 30°C (8 independent leaf samples).

The temperature relations of RNAase are of special interest because this enzyme has been reported to be fairly stable at higher temperatures. Optimum temperature for incubation was found to be around 65°C for pancreatic RNAase (Kunitz, 6). In our experiments with citrus leaves maximum hydrolysis was obtained at a temperature around 70°C (See Figure 1).

Figure 1.

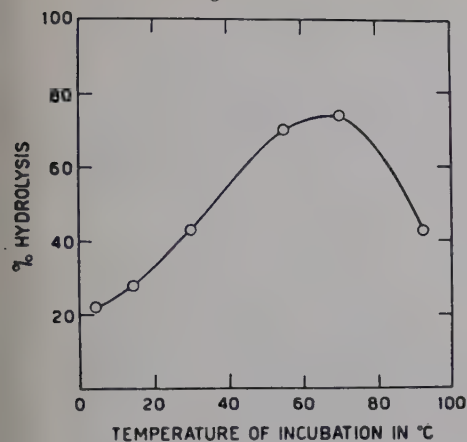


Figure 2.

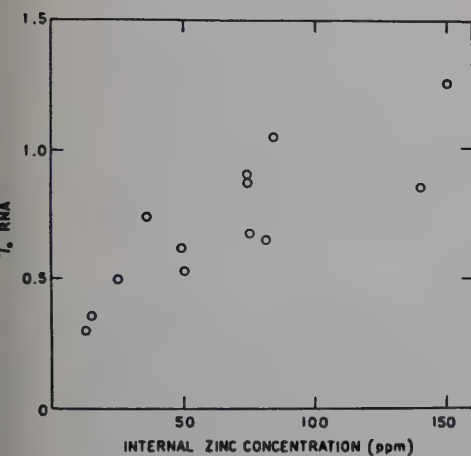
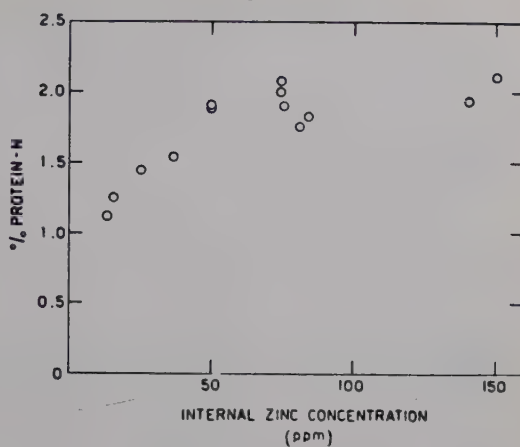


Figure 3.

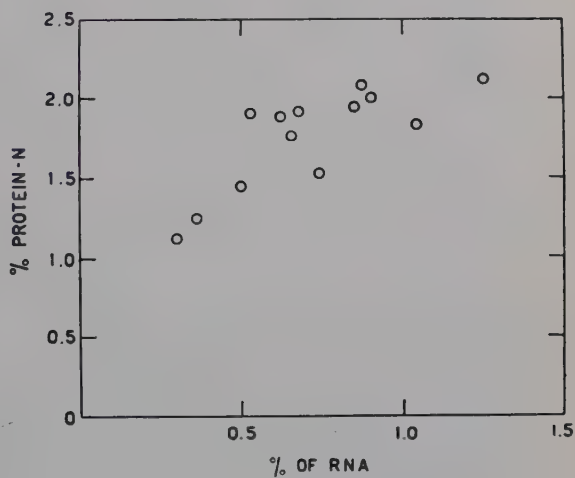


Figure 4.

Figure 1. The effect of different incubation temperatures upon the activity of Citrus RNAase. (Citrate buffer: pH 6.8; time: 3 hours.)

Figure 2. The relation between the levels of zinc and protein in Citrus Leaves.

Figure 3. The relation between the levels of zinc and RNA in Citrus leaves.

Figure 4. Relation between the levels of RNA and protein in Citrus leaves.

Table 2. *Internal zinc concentrations, RNAase activity, and the levels of RNA and different nitrogen fractions in healthy and zinc-deficient Citrus leaves.*

Leaf Type	Zn (ppm)	RNAase (% hydrolysis)	RNA (% dry matter)	Nitrogen (% dry matter)		
				Total	Protein	Soluble ¹
Healthy Leaves	53	41	0.62	2.70	2.08	0.62
Zinc-Deficient Leaves	14	51	0.25	2.66	1.44	1.22

¹ Calculated difference from total and protein nitrogen.

Aqueous solutions of RNAase become slightly inactivated when kept for prolonged periods at higher temperatures (Holden and Pirie, 4). Citrus RNAase too was only slightly inactivated: when the enzymatic extract was kept for one hour at 54°C with a pH of 6.8, it hydrolysed 7 % less of the RNA substrate during the following incubation.

In the following tests, not the optimal temperature for the enzyme activity (70°C), but a more physiological temperature (30°C) was chosen: at this temperature the activities of RNAase from both healthy and zinc-deficient leaves were compared. RNAase extracted from zinc-deficient tissues showed a consistently higher activity than that extracted from healthy leaves. An average difference of 9.4 % in the RNA hydrolysis by enzyme extracts of healthy and zinc-deficient leaves was found. This difference was found to be significant at 0.05 level when tested by "Student's" test.

Testing the internal zinc concentrations of different citrus leaf samples showed that approximately up to 25×10^{-5} gram equivalent (g. equ.) Zn per kg leaf dry matter (16 ppm), RNAase activity was relatively high (usually above 50 % hydrolysis), and the leaves showed the typical mottled deficiency pattern (Table 2). At internal zinc concentrations of 4×10^{-4} g. equ. Zn per kg dry matter (25 ppm) or higher, leaves did not show any visible signs of zinc deficiency (in accordance with accepted standards) and the activity of RNAase was lower.

Another factor which is usually associated with zinc deficiency is protein. At present it is widely assumed that protein synthesis is closely related to RNA metabolism. If we assume that zinc functions through its suppressing effect on RNAase then it might be expected *a priori* that in zinc-deficient leaves the relative levels of protein as well as that of RNA will be lower. This situation was actually found. From Table 2 it becomes apparent that the levels of both protein and RNA are considerably higher in healthy leaves than in the zinc-deficient leaves. It can be further seen that these factors are inversely related to the levels of RNAase.

In order to test more closely the relation between the above variables, zinc was applied externally to healthy leaves. Zinc sulphate, neutralized by lime, was sprayed on healthy leaves at concentrations of 0.5 % and 1 % respectively. Ten days after spraying, leaves were collected, carefully washed as outlined previously, and analysed for zinc, RNAase, RNA, total and protein nitrogen. The soluble nitrogen was obtained by difference. No correlation was found in these experiments between the levels of zinc in healthy leaves and RNAase ($r = +0.301$; r required for significance at 0.05 level: 0.666).

On the other hand the increasing internal zinc concentration is reflected in the level of protein nitrogen (Figure 2).

In Figure 2 zinc concentrations and protein nitrogen were plotted against one another, irrespective of treatments. It is obvious that as zinc concentrations are increased so too is the protein synthesis.

Similar trends were found for the relation between zinc and RNA (Figure 3).

The positive effects of zinc upon protein and RNA synthesis even in healthy leaves are of special interest in the light of recent findings that also in fruit trees protein synthesis is intimately related to RNA (Kessler, 5). The existence of such a relationship can be demonstrated also for citrus leaves, comparing the levels of protein with the corresponding level of RNA (Figure 4). It can be seen from Figure 4 that the levels of protein and RNA parallel each other fairly closely.

Discussion

In the present paper several factors, which were considered to be related to zinc, were tested in citrus leaves. It may be stated quite generally that in zinc-deficient leaves the RNAase activity is higher than in healthy leaves, which contain sufficient zinc. Zinc ions have been reported to be powerful inhibitors of RNAase when added *in vitro* to the reaction mixture at concentrations around 2×10^{-6} M (Holden and Pirie, 4) RNAase extracts from zinc-deficient leaves (containing about 25×10^{-5} g. equ. Zn per kg dry leaf) hydrolyzed about 10 % more substrate than extracts from healthy leaves. Hence it is assumed that one of the functions of zinc is the inhibition of RNAase in the plant. RNAases are phosphodiesterases; other phosphatases too have been reported to show higher activities in zinc-deficient plants (Reed, 8, Robinson, 9), and it may be that zinc is inhibitive to a larger number of different phosphatases. Thus zinc may regulate RNA metabolism through its inhibiting effect upon RNAase. In this respect the relations between zinc, RNA and protein are of special interest.

Today considerable evidence has accumulated that the syntheses of RNA

and protein are very closely related, and any inhibition of RNA synthesis interferes with the formation of protein. In this latter respect the lower activity of RNAase in healthy leaves points *a priori* to higher levels of RNA and protein, a situation which was actually found. It has indeed been demonstrated that proteolysis occurred in zinc-deficient plants (Hoagland, 3, Wood and Sibly, 11) and that the addition of zinc promotes synthesis of protein (Wood and Sibly). In this paper the effect of zinc upon protein synthesis was also demonstrated, increasing concentrations of the latter being accompanied by an increasing level of RNA. This parallelism between the concentration of zinc and RNA was found to be true for deficient as well as healthy leaves where no further correlation exists between the internal zinc concentration and the activity of the extracted RNAase. Up to a certain zinc concentration we must hence assume an effect of zinc upon the formation of RNAase, as the watery enzymatic extracts contain only small concentrations of zinc; the absence of even small amounts of free zinc was further assured by the use of citrate buffer, which is able to bind metals. The incubation was thus carried out in the absence of the inhibiting metal, and the results must be related to the level of RNAase present in the extract from the respective leaf sample. We assume, therefore, that not only the activity, but the total level of RNAase is higher in zinc-deficient leaves, and in these leaves there exists an inverse relation between the zinc concentration and the total level of RNAase. But the situation is different in healthy leaves. In healthy leaves (above approximately 25 ppm Zn) the formation of RNAase remains unaffected by any further increase in the zinc concentration, and even 150 ppm zinc (as found in our experiments) do not suppress further the amount of RNAase below a level which induces about 35 % hydrolysis of the substrate. On the other hand the levels of RNA and protein continue to increase in the healthy leaves parallel with increasing zinc concentrations (Figures 2 and 3). Hence it might be that these higher zinc concentrations suppress *in vivo* the activity of RNAase present in leaves, an assumption which could not as yet be tested directly by experiments. It is thus postulated that increasing zinc on the lower concentration range interferes with the formation of RNAase, while on the higher concentration range it inhibits RNAase activity, leaving RNA to proceed with protein synthesis.

It appears that the above suggested mechanism may be one of the means through which zinc affects protein synthesis while, of course, there might be other mechanisms linking zinc to protein metabolism. At this point one might recall our previous remark that zinc possibly inhibits several phosphatases. Hoagland (3) suggested that zinc acts in phosphorylating processes, and in this respect too, zinc might possibly function through its inhibitive effect upon phosphatases, RNAase being one of them.

Summary

The relation of zinc to RNAase, RNA and protein were investigated in healthy and zinc deficient citrus leaves.

The activity of RNAase is higher in zinc deficient leaves than in healthy ones.

Protein and RNA synthesis are positively related to the level of zinc.

The results are discussed in the light of recent theories with regard to the interaction of RNA and protein synthesis.

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The Phototactic Behaviour of *Chlamydomonas snowiae*

By

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The study of phototaxis in unicellular algae always interested physiologists. As early as 1917 Mast described an action spectrum for the phototaxis of *Chlamydomonas*. More recently Hartshorne (1953) studied the function of the eyespot in the phototaxis of *Chlamydomonas*. Gossel (1957) has reported on the action spectrum of the phototaxis of chlorophyll free *Euglena*. Van Niel's work in the relation between phototaxis and photosynthesis in bacteria (see for example van Niel, 1956 and other literature cited there) has shown how complicated the study of phototaxis in the organism can be. This work is the only one of its kind which clearly related the tactic movement to a definite pigment system and shows its interrelationship with other metabolic processes.

It is of interest therefore to study some aspects of the phototaxis of a green alga, *Chlamydomonas*, whose photosynthetic action spectrum is more or less established, being essentially that of the Chlorophylls *a* and *b*.

Methods and Results

Phototaxis was determined by observing the accumulation of the algae at a small spot on the side of a 5 mm. wide cuvette. The cuvette was enclosed in a light proof case having only one small opening facing the light. The illuminated spot was always below the water-gas interphase. The algae used were *Chlamydomonas snowiae*, isolated from a *Chlorella* mass culture (Mayer and Bamberger, 1958) and identified by Dr. R. Lewin. The algae were grown in the basic medium described by Hutner and Provasoli (1951). Cultures selected for study were tested for phototactic response

Table 1. *Effect of temperature on phototactic response.* Light intensity 110 f.c. white light.

Temperature C°	Length of illumination		
	5 min.	10 min.	45 min.
5	—	—	—
10	—	—	—
15	±	+	+
22	++	++	++
26	++	++	+++
30	++	+++	++++
34	++	++++	++++
30	++	++++	++++

— no taxis ± slight taxis + taxis
 + + + + very marked taxis, algae adhering strongly to surface of cuvettes.

before use. The algae showed a tremendous variability in their phototactic response. They were not always phototactic, and if phototactic, the rapidity of the response varied from day to day.

When taxis was absent, this could be shown to be due to the fact that the algae were in a state of division, several cells being within an envelope of the parent cell. The cause of the difference in speed of response in algae which were visibly motile could not be determined. Age of the culture was one but not the only factor involved. There is evidence for some induction of the tactic movement as slowly responding cultures showed quite rapid response with increasing time of unilateral illumination. This behaviour made an accurate quantitative assessment of phototaxis extremely difficult.

The effect of temperature on the phototactic movement was first studied. A single tactic algal culture was divided and placed in a series of cuvettes. The cuvettes were left in the incubators till temperature equilibrium was reached. They were then shaken and placed in the light proof containers, previously described, facing the light. The extent of the taxis was determined visually by judging the size and intensity of the green colour of the spot on the side of the cuvette. Table 1 shows some of the results obtained. This table shows that the response is a function not only of temperature but also of time. The optimal response lies around 39°C for short periods of time, but at 30—34°C when the exposure is prolonged. The algae also showed some settling.

It is of interest to note that the rate of settling was directly proportional to the temperature being most rapid at 5°C and almost absent at 39°C.

The effect of light intensity on the phototactic response was next determined (Table 2). It will be seen from this table that even very low light intensities cause taxis and the extent of tactic response is also a function of the time of exposure and not merely of the light intensity. An attempt was made

Table 2. *Effect of light intensity on phototactic response. Temperature 26°C.*

Light intensity f. c.	Length of illumination	
	5 min.	10 min.
5	±	±
10	(+)	(+)
20	+	+
50	+	+(+)
100	+(+)	++
200	++	+++
300	++	+++

— no taxis ± (+) + ++ +++ increasing tactic response

to find the light intensities at which negative phototaxis occurs in this species. The time of exposure used was 6 mins. Up to light intensities of 900 f.c. there was strong positive phototaxis. At increasing light intensities, 1000—2000 f.c., the algae moved to the perimeter of the illuminated spot. Marked negative phototaxis became evident only at very high light intensities 2000 f.c. and higher.

It was of interest to determine just how rapidly the algae could respond to light. The ray of light of an Eel Absorptiometer was passed through a diaphragm in order to permit the illumination of a very small area of the cuvette. The cuvette was then placed in the colorimeter, the light switched on and the galvanometer adjusted to 100 % transmission. The decrease in % transmission due to the accumulation of the algae was then followed with time (Table 3). As will be seen, there was a marked response after 30 sec, but as time increased, this turned to be a negative taxis, due to the high light intensity used — 2000 f.c. This was indicated by a rise in % transmission after 4 minutes.

Attempts to make a quantitative estimation of the extent of phototaxis by measuring the change in percent transmission of the culture after part of the algae has moved to the side of the cuvette and adhered there, were unsuccessful. Apparently there was to some extent settling of the algae and adherence to the cuvette side was not always equal.

Table 3. *Change in % transmission of cuvette containing a Chlamydomonas culture when placed in Eel Absorptiometer, the cuvette being masked to have a small area exposed to the light. No filter was used. Temperature 22°C.*

Time in minutes:	0	1/2	1	1 1/2	2	2 1/2	3	3 1/2	4	4 1/2	5
% transmission:	100	96	91	89	87	83	78	73	72	77	80

Table 4. *Phototaxis of Chlamydomonas culture at various wavelengths after 5 mins exposure, light intensity $\frac{1}{2}$ f.c. of the monochromatic light. Temperature 26°C.*

m μ	Response	m μ	Response	m μ	Response
435	—	470	+++	505	+
440	\pm	475	+++	510	+
445	+	480	+++	515	(+)
450	+	485	++	520	\pm
455	+	490	++	525	\pm
460	++	495	++	530	—
465	+++	500	+++		

— no taxis (+) + ++ +++ increasing tactic movement

The action spectrum of the taxis was therefore also determined by visual scoring. The cuvettes were exposed to a beam of light from a monochromator, focused on the side of the cuvette. Preliminary experiments using filters had shown the active light range to be between 400 and 550 m μ . This range was therefore tested at 5 m μ intervals using the same culture for the entire experiment and determining response after 5 mins of exposure. It was somewhat difficult to estimate the exact extent of the response. No marked difference could be observed after 5 min of $\frac{1}{2}$ f.c. monochromatic light in the range 465 m μ —500 m μ . Outside this range the extent of phototaxis dropped rapidly till at the wavelengths of 430 m μ and 530 m μ no taxis was observed (Table 4). Thus there is no evidence for a clear peak in taxis at 500 m μ as previously reported by Mast (1917).

The phototactic behaviour of this algae was not CO₂ dependent. Cultures swept out with a stream of CO₂-free air showed tactic movements identical with that of a normal culture. Thus here the movement was clearly phototaxis and not dependent on the presence of CO₂ (Mayer and Poljakoff-Mayber, 1957).

It is of interest to determine what form of metabolic inhibitor affects phototactic movement. As will be seen from table 5, only 2, 4 di-nitro-phenol (DNP) is effective at low concentrations. This effect was not due to the light absorption of the compound, as normal taxis occurred when light was filtered through the solution at the effective concentration into the tactic culture. 5×10^{-4} M DNP completely inhibited tactic response. The experiments were carried out after preincubation of the culture for 15 minutes with the inhibitor, so as to ensure that the inhibitor did penetrate the cells, to the extent that the cells are permeable to them. After this the tactic response was tested repeatedly for a period of up to one hour without affecting the results.

The pigment system in *Chlamydomonas* according to the literature is

Table 5. *Effective concentration of metabolic inhibitors needed to inhibit phototaxis in Chlamydomonas.*

Inhibitor:	NaCN	NaF	CH ₃ ICOOH	Na ₂ AsO ₃	DNP
Concentration which just does not effect taxis after exposure of algae for 25 mins:	$2 \times 10^{-3}M$	$2 \times 10^{-3}M$	$2 \times 10^{-3}M$	$2 \times 10^{-3}M$	$3 \times 10^{-4}M$

typical of the green algae which closely resembles that of higher plants. As the pigments of the species investigated have not been previously described, it was decided to try and identify the carotenoid pigments.

Chlamydomonas was cultured as usual, and the cells centrifuged down. The wet packed cells were extracted for a few minutes with hot 96 % ethyl alcohol. The extract so obtained was transferred to ether, the alcohol being shaken out with water. The ethyl-ether extract was treated with 30 % KOH in methyl alcohol to saponify the chlorophylls. An excess of petroleum ether, BPt 40—60°C, was added to the residual pigments in the ethyl-ether and these were then separated into two fractions by extraction with 90 % Me.OH. The petrol-ether fraction contained carotenes and the Me.OH fraction contained xanthophylls. These latter were re-extracted into ether.

The absorption spectrum of the two fractions was determined. The fraction containing the carotenes showed peaks at 460 mμ and at 476 mμ. The xanthophylls showed peaks at 440 mμ and 470 mμ and a shoulder at 420—430 mμ. This indicates the pigments to be carotene and luteol respectively. The purity of these pigments was further tested by paper chromatography in various solvents. The carotene extract gave one clear spot in methanol at Rf. 0.64 and in toluene and CCl₄ at the solvent front. One spot was also found when the pigment was examined two dimensionally in combinations of these solvents. The luteol fraction did not appear to be homogenous. In CCl₄ and toluene there was streaking, indicating 3 substances not travelling with the front.

In methanol two spots appeared just below the solvent front. When run two dimensionally at least three spots appeared, one of which corresponded to luteol, one to luteol epoxide while the others could not be identified.

Discussion

It will be seen from the experiments described that the phototactic response is a typical physiological phenomenon which shows temperature dependence. The extent of the response is also determined by the time factor. The findings here differ from those described by Hartshorne, 1953, for *Chlamydomonas Reinhardtii* who found negative taxis to begin above 170 f.c. given for 5 min.

Here negative taxis did not begin till an intensity of 2000 f.c. had been reached. The extent of phototaxis and the speed of the response were determined in some unknown way by the previous history of the culture, *i.e.* age, temperature and culture conditions. As long as the culture was motile as determined by microscopic observation, it also showed eventually phototactic movement. However the extent of this movement is very variable. The experiment using metabolic inhibitors indicates that taxis may depend on a energy supply by oxidative phosphorylation as DNP prevents taxis readily at low concentrations. The other respiratory inhibitors prevent taxis only at very high concentration, when it must be presumed that processes other than respiration are also being affected. In other words, interference somewhere along the oxidative chain is not particularly effective in preventing taxis. Uncoupling of phosphorylation from respiration and also prevention of utilisation of ATP bonds by DNP does stop taxis readily.

The action spectrum of the taxis does not correspond to the absorption spectra of the yellow pigments present in the algae. The action spectrum is much wider than that of the absorption spectra and shows no clear peaks. The peaks previously reported at 500 m μ (Mast, 1917) could not be confirmed. Nishimura and Takamatsu (1957) reported a carotene-protein complex from green leaves whose absorption spectra is markedly shifted to higher wavelength as compared with carotene. This protein-carotene complex has a wider absorption than the carotene. Gossel (1957) relates the action spectrum of *Euglena* to the absorption of the eyespot and Hartshorne (1953) has shown that *Chlamydomonas* without eyespots is almost not phototactic. Thus the function of the eyespot in phototaxis is fairly well established. Obviously, however, no known yellow plant pigment can account for the action spectrum. If, however, the carotene absorption spectrum is suitably modified by complexing with protein, then this may account for some of the observed phenomena.

Summary

The phototactic behaviour of *Chlamydomonas snowiae* is described. It is shown to be temperature dependent and to involve processes of oxidative phosphorylation. The action spectrum shows a plateau between 460—500 m μ . This cannot be related to the carotenoid pigments as such, present in the alga. The possibility that a carotene-protein complex is responsible for the action spectrum is discussed.

Our thanks are due to Dr. R. Lewin for identification of the *Chlamydomonas* species. Stimulating discussion with Prof. C. B. van Niel brought us to this subject and we are therefore greatly indebted to him.

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The Effect of Gibberellic Acid on Shoot Growth of Cupid Sweet Peas

By

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Introduction

The effect of gibberellic acid (GA) on the shoot growth of a plant (garden pea, *Pisum sativum*) with a single unbranched stem has already been quantitatively studied (Brian and Hemming, 1955). Its effects on the growth of plants with a naturally bushy or branched habit of growth are known to be more complex, since effects on apical dominance are involved. The present paper describes in quantitative terms the effect of GA on the shoot growth of such a plant — the dwarf "Cupid" sweet pea (*Lathyrus odoratus*). The opportunity was taken to compare the effects of single initial doses with continued GA applications. Some cursory observations on flowering and seed production were also made, though these were secondary to the main purpose of the investigation.

Materials and Methods

Experimental plants. — "Cupid" sweet pea seed was obtained from Thompson and Morgan (Ipswich) Ltd., Ipswich. These were sown in 7 inch pots in John Innes Compost No. 2 on 10th April, 1957, and the seedlings thinned out to leave four plants in each pot. Four pots of 4 plants were used for each of the treatments described below (with some extra plants as described under "Leaf Measurements"). The plants were treated on 30th April, 1957, when they were about 35 mm. high, with 3—4 internodes in the unbranched main axis. All were grown in an unheated glass-house and supported with stakes as necessary.

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Application of gibberellic acid. — GA was applied in 2 μ l. ethanol to the apical bud by the method previously described (Brian and Hemming, 1955). The treatments included were single initial treatments with 1, 10 and 100 μ g. GA and weekly doses of 1 μ g. GA. The "untreated" plants received an initial dose of 2 μ l. ethanol alone, though preliminary experiments had indicated that even repeated doses of ethanol of this size had no effect on plant growth.

Stem-measurements. — All internodes were measured at the beginning of the experiment and thereafter at 14 day intervals until 70 days had elapsed; individual records were kept for each internode. The point of origin of each branch was recorded and each branch was identified by coloured plastic rings. Branches less than 10 mm. in length were ignored.

Leaf-area measurements. — A number of extra pots of each treatment were provided and on three occasions (viz. 27, 51 and 64 days after the first treatment) four plants were sacrificed, all leaves being removed and records of their size and shape made photographically by the "Ozalid" [Ozalid Co. Ltd., London Wall, London, E.C.2] positive photoprint process. Leaf area estimations were made by cutting out and weighing these photographic silhouettes. This sample of 4 plants was undoubtedly too small to detect small differences in leaf-growth, but the task of recording would have been too great if a larger sample had been taken.

Some observations on flower and seed production, described in the text, were made towards the end of the experiment.

Statistical analysis. — Where considered necessary data were analysed by standard analysis of variance methods, and results of such analyses are presented in the form of tests of significance of differences, using the *t* test.

Results

Growth of stem

The effects of GA on stem length are presented in Table 1. All treatments increased main axis length. In single-dose treatments the size of the response increased with size of dose. As the dose was increased growth rate increased (Figure 1) and an increased growth rate was maintained for a longer period with high doses than with low doses. Weekly applications of 1 μ g. GA induced greater main axis extension by the end of the experimental period (70 days) than an initial dose of 100 μ g.; after only four applications of 1 μ g. the weekly treatment had induced greater extension than an initial dose of 10 μ g. These trends are also all reflected in the data for main axis internodes numbers (Table 2).

The effects on branch growth were more complex. For the first 14 days of the experiment all GA treatments reduced branch extension, whether measured as total branch length (Table 1, Figure 2), number of internodes in branches (Table 2) or number of branches (Table 3), the most marked inhibition of branching being produced by the 100 μ g. initial dose of GA.

Table 1. *Effect of GA treatments on mean length of stem (mm.) in main axis and branches.*

MAIN AXIS

GA treatment	Days after initial treatment				
	14	28	42	56	70
None	118	136	152	158	158
1 µg. initial	150	186	212	236	257
10 µg. initial	207	310	375	424	469
100 µg. initial	268	554	805	960	1,038
1 µg. weekly	173	376	733	1,002	1,208

BRANCHES

GA treatment	Days after initial treatment				
	14	28	42	56	70
None	34	116	324	649	944
1 µg. initial	27	106	330	614	941
10 µg. initial	21	123	391	666	991
100 µg. initial	3	172	640	906	1,122
1 µg. weekly	23	180	491	638	849

MAIN AXIS AND BRANCHES

GA treatment	Days after initial treatment				
	14	28	42	56	70
None	152	252	476	807	1,102
1 µg. initial	177	282	532	850	1,198
10 µg. initial	228	433	766	1,090	1,460
100 µg. initial	271	726	1,445	1,866	2,160
1 µg. weekly	196	556	1,224	1,640	2,057

In untreated plants three categories of branches were produced: — I, main branches arising from axils of leaves of the main axis; II, branches arising from lower nodes of main branches; III, short branches developing from category II branches. The development of these branches is described statistically in Table 3. Treatment with GA completely suppressed all category III branches and delayed the appearance of category II branches. During the first 14 days of the experiment the various GA treatments differed in effect only quantitatively, but later certain qualitative differences developed.

The smaller single-dose treatments (1 and 10 µg. GA) had little further effect on branch growth after 14 days, so that at the end of the experiment plants which had been so treated differed little from untreated plants in number of branches, number of branch internodes or total branch length; nevertheless, as a result of such treatments a greater proportion of branch growth was directed into category I branches (Table 4).

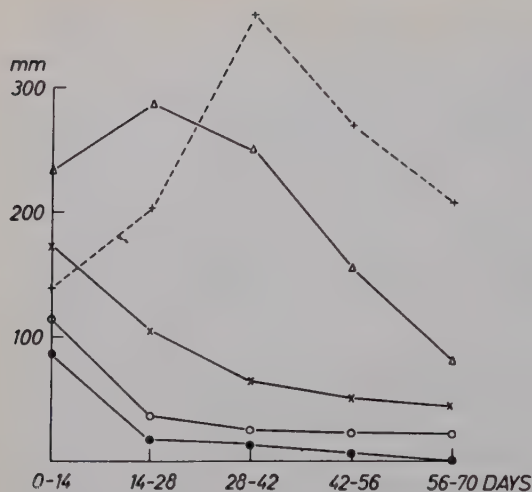


Figure 1. Mean rate of extension (mm./14 days) of main axis.

- Untreated
- 1 µg. G.A. initial dose
- × 10 µg. G.A. initial dose
- △ 100 µg. G.A. initial dose
- + 1 µg. G.A. weekly

An initial dose of 100 µg. GA had more far-reaching effects. Though for 14 days it reduced branch length, thereafter it resulted in quite considerably increased branch length (Table 1). This was not accompanied by an increase in the number of internodes; on the contrary, the number of branch internodes was reduced (Table 2), as were the number of branches (Table 3). The increase in branch length was confined to category I branches (Table 4).

The weekly 1 µg. dose, after the initial check of branch growth, continued to inhibit formation of new branches and internodes until 42 days after the beginning of treatment, though during that period total branch length moved slightly ahead of that of untreated plants. After that, a number of small branches developed near the top of the main axis, so that eventually the total number of branches exceeded that of untreated plants (Table 3), but these were almost exclusively category I branches. This increase in number of branches was not accompanied by a comparable increase in total branch length and the number of branch internodes remained significantly lower than that in untreated plants throughout the experiment.

Considering the stem system as a whole at the end of the experiment, the responses to GA treatment may be summarised as follows: —

- (1) All treatments increased total stem length; if the single-dose treatments are compared, the greater the dose the greater the increase in stem length; 10 weekly 1 µg. doses increased stem length nearly as much as an initial 100 µg. dose and much more than an initial 10 µg. dose.

Table 2. *Effect of GA treatments on mean number of internodes in main axis and branches.*

MAIN AXIS

GA treatment	Days after initial treatment				
	14	28	42	56	70
None	8.9	12.2	15.6	16.5	17.0
1 μ g. initial	10.0	13.3	17.7	22.2	25.1
10 μ g. initial	10.5	15.1	20.0	25.4	29.1
100 μ g. initial	10.2	16.8	23.4	29.7	34.7
1 μ g. weekly	9.7	15.7	23.0	30.4	35.6
Significant difference ($P=.05$)	0.6	0.9	1.6	2.0	2.6
Significant difference ($P=.01$)	0.8	1.2	2.2	2.7	3.5

BRANCHES

GA treatment	Days after initial treatment				
	14	28	42	56	70
None	4.7	15.9	44.7	72.3	96.4
1 μ g. initial	2.8	13.6	49.6	81.3	101.3
10 μ g. initial	2.4	15.6	44.5	73.7	101.8
100 μ g. initial	0.2	7.1	17.7	31.2	58.0
1 μ g. weekly	2.6	14.8	25.7	40.3	74.2
Significant difference ($P=.05$)	2.2	5.1	9.4	16.3	20.0
Significant difference ($P=.01$)	3.0	6.8	12.4	21.8	26.9

MAIN AXIS AND BRANCHES

GA treatment	Days after initial treatment				
	14	20	42	56	70
None	13.6	28.1	60.3	88.8	113.4
1 μ g. initial	12.8	26.9	67.3	103.5	126.4
10 μ g. initial	12.9	30.7	64.5	99.1	130.9
100 μ g. initial	10.4	23.9	41.1	60.9	92.7
1 μ g. weekly	12.3	30.5	48.7	70.7	109.8
Significant difference ($P=.05$)	2.3	4.3	9.5	16.2	20.4
Significant difference ($P=.01$)	3.1	5.8	12.7	21.7	27.4

- (2) No treatments significantly increased the total number of internodes formed; an initial dose of 100 μ g. GA decreased the number of internodes formed, and other treatments did not affect internode number.
- (3) The only treatments which ultimately affected the number of branches produced were the initial 100 μ g. dose, which reduced branching, and the weekly 1 μ g. dose, which increased branch numbers.

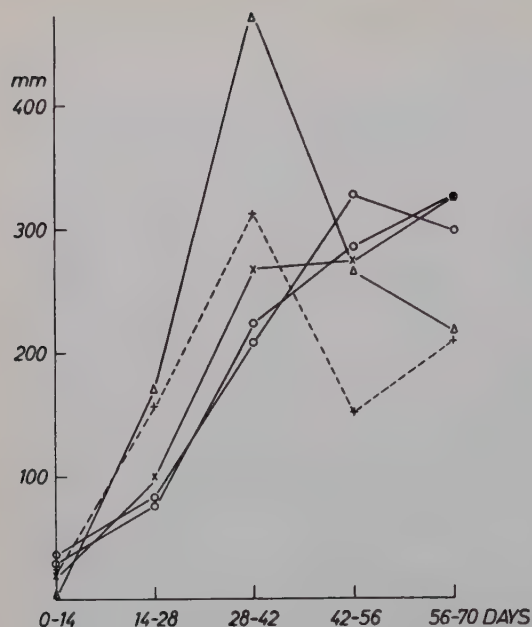


Figure 2. Mean rate of extension (mm./14 days) of branches. [Total increment of all branches of a single plant.]

Table 3. Effect of GA treatments on mean number of branches produced [For description of branch categories see text; total branch numbers only have been statistically analysed].

GA treatment	Branch category	Days after initial treatment				
		14	28	42	56	70
None	I	1.9	3.6	5.1	5.1	5.1
	II	—	0.7	1.7	1.9	2.6
	III	—	—	—	—	0.2
	Total	1.9	4.3	5.8	7.0	7.9
1 µg. initial	I	1.7	3.9	7.2	7.2	7.2
	II	—	0.5	1.2	1.2	1.5
	Total	1.7	4.4	8.4	8.4	8.7
10 µg. initial	I	0.9	3.9	6.6	6.6	7.2
	II	—	0.2	1.0	1.0	1.4
	Total	0.9 ²	4.1	7.6	7.6	8.6
100 µg. initial	I	0.4	1.8	2.1	4.8	4.8
	II	—	—	0.3	0.7	0.9
	Total	0.4 ²	1.8 ²	2.4 ²	5.5	5.7 ¹
1 µg. weekly	I	1.4	2.7	2.9	9.3	10.7
	II	—	0.2	0.4	0.8	0.9
	Total	1.4	2.9 ²	3.3 ²	10.1 ²	11.6 ²

¹ Significantly different from untreated at 5 % level of probability.

² „ „ „ „ „ 1 % „ „ „

Table 4. *Effect of GA on stem length (mm.) of main axis and various categories of branch measured 70 days after initial treatment.*

GA treatment	Branch category			All branches	Main axis	Total
	I	II	III			
None	716	224	4	944	158	1.102
1 µg. initial	858	83	0	941	257	1.198
10 µg. initial	903	88	0	991	469	1.460
100 µg. initial	1.011	111	0	1.122	1.038	2.160
1 µg. weekly	829	20	0	849	1.208	2.057

- (4) These general effects on growth were accompanied by a marked alteration in the balance of growth between main axis and branches and between branches of various categories and positions; this is described in greater detail below.

Effects on plant habit

As already mentioned, untreated plants had a complex branching habit. Typically, vigorous branches arose in the axils of the two scale-leaves (*i.e.* at nodes 2 and 3, if the cotyledonary node is counted as node 1) about a week after the beginning of the experiment. These extended rapidly and themselves branched. During this period growth of the main axis virtually ceased. Somewhat later further vigorous branches usually developed at nodes 2 and 3 of the main axis, and these too themselves branched. A further branch sometimes developed at node 3 of the main axis but this, when present, remained small. The plant habit is illustrated semi-diagrammatically in Figure 3 a.

Plants given an initial 1 µg. dose of GA usually produced only one strong branch at nodes 2 and 3 of the main axis; the second branches at these nodes, though usually formed, remained much shorter than the first ones. Branching at nodes 3—6 of the main axis was frequent and, of course, the main axis extended more than in untreated plants (Figure 3 b). After an initial 10 µg. dose the main axis extended still more obviously; branching frequently occurred at nodes 2—7 of the main axis, but there was a tendency to concentrate growth in single branches at nodes 2 and 3 (Figure 3 c). An initial 100 µg. dose carried this tendency still further so that growth was concentrated almost exclusively in a long main axis and long single branches at nodes 2 and 3. Towards the end of the experiment a few short branches usually developed near the apex of the main axis, in the region of nodes 20—25 (Figure 3 d). The weekly treatment concentrated growth in the main axis; branches arose at nodes 2 and 3 but failed to extend much. Towards the end

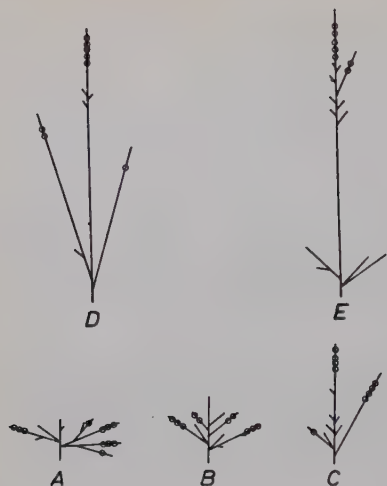


Figure 3. *Generalised representations of plant habit at 70 days: (a) untreated, (b) $\mu\text{g. GA. initial dose}$, (c) $10 \mu\text{g. GA. initial dose}$, (d) $100 \mu\text{g. GA. initial dose}$, (e) $1 \mu\text{g. GA. weekly}$. [Position of inflorescences with open flowers represented by open circles.]*

of the experimental period numerous small branches were produced near the apex of the main axis between nodes 20 and 30 (Figure 3 e). Thus by altering the treatment régime a number of distinct modifications of plant habit were obtained.

Persistence of the effect of a single dose of GA

We have shown elsewhere (Brian and Hemming, 1955) that, in the garden pea, the main effect of increasing the size of a single GA application is to prolong the period during which growth is accelerated. With small doses the growth rate eventually returned to that of untreated plants, and it was concluded that GA is lost either by involvement in metabolic processes concerned in growth or by some other process of degradation. The results now obtained with Cupid sweet peas are generally similar; exact comparisons between the two plant species are not possible since in the experiments now described a higher dose range was studied over a much longer period, with fewer observations.

The growth rate of the main axis of untreated plants declined throughout the experiment and during the final 14-day period (56—70 days after the beginning of the experiment) extension ceased (Figure 1). Initial doses of 1 and $10 \mu\text{g. GA}$ increased growth rate during the period 0-14 days after treatment and thereafter the growth rate declined. However, though it fell to a low level it never completely ceased, and we must conclude that an initial dose of only $1 \mu\text{g. GA}$ continued to exert an effect on the activity of the apical bud of the

main axis during the period 56—70 days after treatment. After an initial dose of 100 μ g. GA the growth rate was maintained at a high level for 42 days after treatment, thereafter declining. Plants receiving a weekly dose of 1 μ g. weekly reached a maximum growth rate during the period 28—42 days after the first application; at that time the main axes of such plants extended more rapidly than plants receiving a single 100 μ g. dose, but after the 42nd day the rate of extension declined rapidly. Thus while the fall in growth rate during the latter part of the experiment was probably partly due to loss of GA, the fact that continued dosage failed to prevent the fall indicates that it was partly due to endogenous causes unconnected with inactivation of GA. In fact the plants became obviously vegetatively mature during that period.

The rate of branch stem extension (Figure 2) of untreated plants continued to increase until 56 days after the beginning of the experiment, thereafter falling slightly. GA doses of 1 μ g. and 10 μ g. had little effect on branch growth rate after the first 14 days. The branch growth rate of plants given an initial 100 μ g. dose or treated weekly with 1 μ g. GA, after an initial check, was higher than that of untreated plants in the period 14—42 days, but thereafter fell to a level below that of untreated plants. Thus though the growth rate of all stems (main axis + all branches) in the final 14-day period of the experiment was fairly close in untreated and treated plants (see Table 1), the effects of the treatments were still clearly evident in the tendency to direct growth into the main axis and certain specific branches.

Effects of flower-formation on internode extension

Lower internodes in the main axis of untreated plants averaged 10—14 mm. in length, but upper internodes were shorter, rarely exceeding 5 mm. in length. The same decrease in internode length could be traced in the larger branches, the first four or five internodes being 12—14 mm. long, subsequent ones 7—9 mm. Flowers were borne near the apex of such branches and it was very noticeable that internodes immediately below nodes bearing inflorescences were usually at least 20 mm. long, i.e. more than double the length of immediately preceding internodes bearing no inflorescences. The higher GA doses tended to mask this difference, all internodes being much longer than in untreated plants. Immature seeds contain relatively high concentrations of gibberellin-like hormones (Phinney, West, Ritzel and Neely, 1957; Radley, 1958; MacMillan and Suter, 1958); the increased extension of the internodes below inflorescences is possibly due to production of such hormones in the developing flowers.

Table 5. *Effect of GA treatments on total leaf area per plant (cm²) and mean leaf-size (mm²).*

TOTAL LEAF AREA PER PLANT

GA treatment	Days after initial treatment		
	27	51	64
None	6.1	36.1	59.8
1 µg. initial	10.6	44.9	68.2
10 µg. initial	11.4	38.7	57.1
100 µg. initial	7.8	30.4	48.8
1 µg. weekly	12.6	41.3	65.8
Significant difference (P=.05)	3.2	12.1	20.0
Significant difference (P=.01)	4.2	16.7	27.8

MEAN LEAF SIZE

GA treatment	Days after initial treatment		
	27	51	64
None	283	504	633
1 µg. initial	375	547	680
10 µg. initial	433	562	568
100 µg. initial	573	692	544
1 µg. weekly	438	795	530
Significant difference (P=.05)	128	151	180
Significant difference (P=.01)	177	209	249

Growth of leaves

We have seen that the total number of internodes per plant was reduced by the 100 µg. dose of gibberellic acid but that other treatments had little effect on internode number (Table 2). This was borne out by leaf counts on those plants used for leaf-area measurement. A noticeable effect of GA treatment of Cupid sweet peas is to change the shape of the leaflets from ovate to lanceolate (Brian and Grove, 1957). In this experiment the change of leaf shape was most noticeable on the plants treated initially with 100 µg. GA or weekly with 1 µg.; the effect of the smaller doses was rapidly transient. Associated with the change in shape was an increase in leaf-area (Table 5). The sample used for leaf-area measurements was too small for the detection of small differences and effects on total leaf-area per plant were only detected in the plants measured 27 days after treatment; total leaf-area was at that time increased by the 1 and 10 µg. initial doses and the weekly 1 µg. dose. The failure of the 100 µg. dose to increase total leaf-area was due to the reduction in number of leaves, since mean leaf-area (mean area of a single leaf) was greatly increased. Indeed mean leaf-area was still noticeably greater

Table 6. *Effect of GA treatments on mean number of inflorescences per plant and on mean length of inflorescence stalks (mm.).*

GA treatment	All inflorescences	Inflorescences with open flowers	Length of inflorescence stalks
None	22.4	11.2	41.4
1 µg. initial	23.4	9.2	44.3
10 µg. initial	26.3	9.1	48.7
100 µg. initial	22.3	7.1	56.8
1 µg. weekly	31.2	7.2	93.7
Significant difference (P=.05)	6.4	2.9	
Significant difference (P=.01)	8.5	3.7	

in plants treated with 100 µg. GA, or weekly 1 µg. doses, 51 days after the first application; the failure to maintain this increased average leaf-size until the end of the experiment was mainly due to a rapid increase in branch internodes towards the end of the experiment (Table 2); these new branches, near the apex of the main axis, bore very small leaves.

Flowering and fruiting

Flower buds were first observed between 42 and 56 days after the beginning of the experiment. In untreated plants no flowers developed on the main axis, but were borne on branches of categories I and II (see Figure 3). The 1 µg. dose of GA had little effect on the position where flowers were borne, but plants receiving higher doses bore inflorescences on the main axis, and in plants treated with weekly doses of 1 µg. flowering was almost confined to the main axis.

A count of inflorescences was made at the conclusion of the experiment (70 days). None of the single dose treatments affected the number of inflorescences, but their number was significantly increased by the weekly 1 µg. treatment. There was a tendency for GA treatments to delay the opening of flowers, most noticeable with those doses having the greatest effect on vegetative growth (Table 6). GA treatment increased the length of inflorescence stalks. Though treatment ceased at this stage and no further detailed measurements were made, a further count of inflorescences was made 90 days after the first GA treatment, and again it was noted that the weekly 1 µg. dose of GA had increased the number of flower-buds visible, though the effect was by then less marked (a 20 % increase as compared with a 50 % increase at 70 days). It was very noticeable at this stage that dehiscence of flowers was much reduced by GA treatment and this resulted in more seed pods devel-

Table 7. *Effect of GA treatments on yield of seed* [Figures given in first two columns are totals for 16 plants].

GA treatment	No. of seeds	Weight of seeds (g.)	Mean weight per seed (mg.)	Mean diameter of seeds (mm.)
None	354	26.2	72	4.6
1 μ g. initial	497	39.8	80	4.9
10 μ g. initial	474	46.0	97	5.3
100 μ g. initial	805	83.5	104	5.4
1 μ g. weekly	675	73.6	109	5.5

oping. The plants were therefore kept growing in the glasshouse until seed had matured. Details of the seed harvested 24 weeks after the beginning of the experiment are given in Table 7. All GA treatments increased the number and weight of seeds produced; surprisingly, there was also a considerable increase in seed size. Unfortunately the seed was bulked from all plants in a single treatment, and no statistical analysis was possible; nevertheless it seems highly probable that the effects observed were real.

All seed germinated normally and produced normal dwarfed plants, i.e. there was no "carry-over" of GA or any effect on the genotype.

Discussion

Apical dominance

All the GA treatments increased main axis extension, and, in so far as they channelled a greater proportion of total stem growth into the main axis, it may be said that they enhanced apical dominance. But apical dominance was never complete. The nearest approach to complete apical dominance resulted from weekly applications of 1 μ g. GA; it seems likely that an even more complete suppression of branching would have been achieved by a large (10—100 μ g.) initial dose followed by weekly 1 μ g. doses. The means by which GA maintains a functional apical bud in the main axis is at present completely obscure. Eventually, even with continued treatment, the activity of the apical bud declined and extension of the main axis slowed down. Thus some of the growth-retarding changes in the apical bud can be reversed by GA but others cannot.

The remarkable difference in habit (Figure 3) between plants treated with a single dose of 100 μ g. GA and those treated weekly with 1 μ g., raises the possibility of usefully controlling growth habit by GA treatment instead of

by the more conventional practice of pruning or removal of buds. It seems possible that still other patterns of growth might have been achieved by timing the GA applications differently or perhaps by application to other parts of the shoot system than the apical bud.

These results confirm our earlier view (Brian and Hemming, 1957) that the net effect of GA applications on branching depends on a balance between two conflicting tendencies, a tendency to reinforce the dominance of properly functioning apical buds and a tendency to accelerate development of lateral buds which have been released from inhibition, whether from natural causes or as a result of excision of an apical bud or interference with its activity by application of maleic hydrazide. Wickson and Thimann (1958) have also presented evidence that GA stimulates development of lateral buds only if they have already been released from inhibition. The different patterns of growth observed in these experiments with Cupid sweet peas can probably be attributed to differences in the balance of the two tendencies mentioned above.

Effect of GA on seed yield

It is unfortunate that sufficient data were not collected to explain with greater certainty the increased seed yield. The increased number of seeds harvested after GA treatment is probably a result of reduced flower bud-drop, a trouble to which the Cupid sweet pea is very prone, but other possible mechanisms cannot be ruled out.

The increased seed-size was quite unexpected, but a tentative explanation can be offered. It can now be regarded as certain that the gibberellins are natural plant hormones; the presence in plants of hormones with gibberellin-like physiological properties has been demonstrated (Phinney *et al.*, 1957; Radley, 1958) and MacMillan and Suter (1958) have isolated gibberellin A₁ from immature runner-bean seeds. These gibberellin-like hormones are, together with auxins, particularly abundant in young seeds; if, as seems likely, their presence is necessary for proper maturation of the seeds, the increased growth of Cupid sweet pea seeds in response to GA may be due to a failure to produce sufficient endogenous hormones of this type. Certainly the highly dwarfed vegetative growth of this plant is suggestive of such a failure. It must be admitted, however, that seeds of dwarf garden peas develop normally and their number and size are not enhanced by GA treatment. A study of the gibberellin content of garden pea and Cupid sweet pea seeds during their development should clarify this situation.

Summary

- (1) The total stem length, in main axis and branches, of Cupid sweet peas, was increased by gibberellic acid (GA) treatment. The effect of 10 weekly applications of 1 μ g. GA was much greater than that of a single initial dose of 10 μ g., and nearly as great as that of an initial 100 μ g. dose. The total number of internodes formed was not increased; an initial dose of 100 μ g. GA significantly reduced the total number of internodes formed.
- (2) At first, all GA treatments inhibited branch formation and extension. Subsequently only two treatments influenced the number of branches formed; an initial 100 μ g. dose reduced branching, and weekly application of 1 μ g. GA increased the number of branches. Nevertheless all GA treatments simplified the branching system and directed a greater proportion of the growth into the main axis. Consequently the plant habit was much modified. Untreated plants were short and highly branched, with secondary and tertiary branches developing on the main branches. A single 100 μ g. dose of GA directed growth into a main axis and two long branches arising from nodes 2 and 3 of the main axis. Weekly treatment with 1 μ g. GA confined growth almost exclusively to the main axis.
- (3) All GA treatments, but particularly a single 100 μ g. dose and weekly doses of 1 μ g., increased mean leaf-size, though the total leaf area of a plant was only transiently increased.
- (4) GA treatment slightly delayed the opening of flowers. Weekly application of 1 μ g. GA increased the number of flower buds visible on the two occasions when counts were made. All GA treatments increased the number of seeds produced and increased mean seed-weight. Possible causes of this increased seed yield are discussed.

We are indebted to Professor S. C Harland for suggesting the Cupid sweet pea as suitable experimental material. We wish to thank Mr J. H. P. Petty and Mr. P. T. Richmond for painstaking assistance with the many measurements involved in this investigation.

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The Effect of Cations and Anions on the Respiration Rate of the Brown Alga, *Hormosira banksii*

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Introduction

The emergent brown alga *Hormosira banksii* takes the form of chains of hollow bladders containing sea water, which are extremely tolerant of dehydration. As water is lost during aerial exposure, the salt concentration of the contained seawater increases and simultaneously the rate of respiration rises until a maximum is reached after 20 % of the tissue water has been lost. This enhanced oxygen uptake has also been induced by increasing the concentration of the solution bathing the tissue, as well as by removing the internal fluid and injecting concentrated seawater (Bergquist 1957).

Some preliminary results suggested that when the salt concentration was increased, the respiratory response appeared to be linked to the specific cationic rather than anionic groups of the mixtures used to increase the ionic concentration. Blinks (1951) has remarked upon the resistance to dehydration shown by *Valonia* when an adequate supply of potassium ions was maintained for accumulation (presumably by energy dependent processes), and Epstein (1954) has also observed stimulation of oxygen uptake of excised barley roots when exposed to K^+ ions adsorbed on synthetic cation exchangers. The purpose of this communication is to report the effect on the rate of oxygen uptake by *Hormosira* tissue exposed to cations (K^+ and Na^+) and anions (Cl^-) adsorbed to the appropriate exchangers.

Methods

Carefully matched vesicles from mature vigorous plants of *Hormosira* were sliced in half and briefly washed with autoclaved sea water. Washing the tissue for long periods (up to 168 hours) in frequently renewed sea water did not alter the response apart from causing a slow decline of the rate of oxygen consumption. Oxygen uptake at 30°C was determined by conventional Warburg manometry using a shaking rate of 120/minute with a 3 cm. excursion.

If buffers containing KH_2PO_4 are avoided, the respiration rate of the tissue is not markedly affected by pH within the range 4.5—9.5. Potassium dihydrogen phosphate buffer was found to stimulate oxygen uptake markedly. In contrast, citrate-disodium phosphate buffer, despite its high concentration of a Krebs-cycle intermediate, only slightly stimulates respiration.

To obtain the necessary rigid control of the ionic environment it was in some cases necessary to use artificial sea water. Allen's sea water (Allen 1914) was used at pH 5.0 with reduced Ca and Mg content, the cation concentration being maintained by the Na of the buffer. Other experiments were performed using natural sea water, acidified and unbuffered at pH 5.0 (Table 1) and buffered with Tris (hydroxymethyl)-aminomethane (Tris) at pH 8.0 and pH 9.4. No experiments were carried out in unbuffered seawater at high pH's, since there was too rapid a drift in the pH under such conditions.

In order to increase the concentration of one given ion during the course of the experiment, without increasing the concentration of any other mobile ion, use has been made of various ion exchange resins. These are highly polymeric, tightly cross-linked structures containing polar groups whose charges are balanced by mobile ions (Kunin and Myers 1950). We may therefore consider the cation exchangers as electrolytes with a simple diffusible cation and an enormous, non-diffusible anion. Similarly, the anion exchanger has a diffusible anion and a non-diffusible cation. The cation resin selected was Amberlite IRC-50, a weakly acidic carboxylic acid-type exchanger, which is readily dissociable below pH 7.0, so that it is entirely in the acid form at pH 3.5. The anion resin used was Amberlite IR-4B, weakly basic and readily dissociable above pH 7.0. It must be noted that these resins have been used in a different manner to that of Epstein (1954). He used his weakly acidic resin at pH 8.0, in the slightly dissociated state, and relied on the ability of the barley roots to take up readily mineral ions adsorbed in exchangeable form (*i.e.* adsorbed onto the resin as if on a soil particle). It was considered unlikely that this would be so for marine algae, and hence the weakly acidic resin was used at low pH so that the exchanger existed largely in the ionised form, releasing cations.

The resins were pretreated in the following manner. For experiments using increased concentrations of K^+ or Na^+ , the cation resin Amberlite IRC-50 was titrated with KOH or NaOH, backwashed and drained. Since no indication was available of the actual exchange capacity of the resin under the experimental conditions the amount of resin was determined by trial, the resulting ionic concentration being determined by flame photometry. The potassium ion concentrations in contact with the tissue were 58 $\mu\text{equiv./ml.}$ at pH 5.0, and 14.5 $\mu\text{equiv./ml.}$ at pH 8.0. With sodium ions, the respective concentrations were 692 $\mu\text{equiv./ml.}$ and 485 $\mu\text{equiv./ml.}$

The anion exchanger, Amberlite IR-4B was titrated and backwashed with HCl and the amounts required determined empirically as before. The chloride concentration was estimated by potentiometric AgNO_3 titration. The capacity of the War-

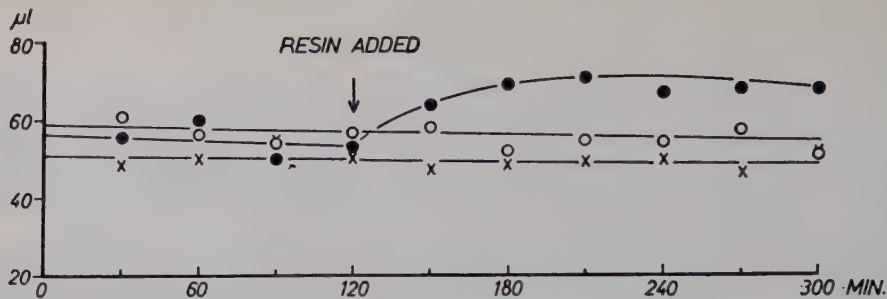


Figure 1. Effect on respiratory oxygen consumption of added K^+ ions. Citrate-buffered artificial seawater, pH 5.0. K^+ = 58 μ equiv./ml. Diacritic symbols: \circ — control, \bullet — K^+ -IRC-50 added, \times — H^+ -IRC-50 added. On the ordinate μ l. O_2 /g. fresh weight.

burg vessel sidearms limited the amount of resin which could be added and at the experimental pH (9.4), this in turn limited the final chloride concentration to 670 μ equiv./ml. for single arm vessels, and 703 μ equiv./ml. for two arm vessels. This latter concentration is very close to values for the chloride ion in the bladder fluid at the maximal rate of oxygen uptake on dehydration.

Results

At pH 5.0 there was an obvious stimulation of oxygen uptake when the K^+ form of the resin was added (Figure 1, Table 1), although this is not as great as that induced when modified sea water with increased K^+ is used (approximately 42 %). The H^+ form of the resin evokes no respiratory response at either pH. At pH 8.0 (Figure 2) there is a slight but temporary stimulation when the tissue is exposed to the K^+ -IRC-50. Addition of Na^+ as the exchangeable cation adsorbed onto IRC-50 evoked no measurable response, either at pH 5.0 or 8.0 (Table 1).

Addition of the anion exchanger (Figure 3) at both concentrations of chloride results in a marked inhibition of oxygen uptake. The OH^- form of the resin evokes no response.

Discussion

These results indicate that at least part of the respiration stimulated by high salt concentrations may be due to the influence of the K^+ ions, and it is unlikely that increased Na^+ concentrations are responsible. Scott and Hayward (1953, 1954, 1955) have provided evidence for separate pathways of Na^+ and K^+ regulation in the marine chlorophycean *Ulva lactuca*. Results,

Table 1. *Effect of cations adsorbed on synthetic exchange resins added to tissue respiring in sea water — data in terms of percentage increase or decrease.*

Substrate	Cation resin added	Cation added		Control	
		Respiratory increase or decrease on addition of resin	Replicates	Respiratory increase or decrease on addition of resin	Replicates
Acidified, natural seawater, pH 5.0	K ⁺ -IRC-50 K ⁺ =58 µequiv./ml.	+ 34.2 %	5	+ 3.4 %	4
Citrate-buffered artificial seawater, pH 5.0	Na ⁺ -IRC-50 Na ⁺ =692 µequiv./ml.	— 1.8 %	6	— 2.5 %	4
Acidified natural seawater, pH 5.0	Na ⁺ -IRC-50 Na ⁺ =440 µequiv./ml.	+ 3.0 %	6	+ 1.5 %	4
Tris-buffered natural seawater, pH 8.0	Na ⁺ -IRC-50 Na ⁺ =485 µequiv./ml.	— 1.0 %	5	+ 1.4 %	4

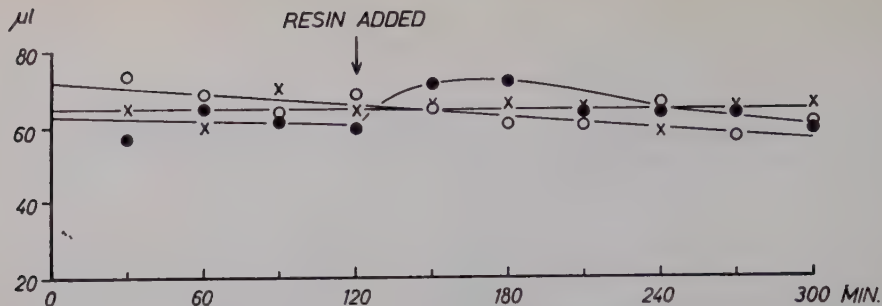


Figure 2. Effect on respiratory oxygen consumption of added K^+ ions. Tris-buffered artificial seawater, pH 8.0. $K^+ = 14.5$ μ equiv./ml. Diacritic symbols: \circ — control, \bullet — K^+ -IRC-50 added, \times — H^+ -IRC-50 added. Ordinate as in Figure 1.

which have been obtained by the writer using similar techniques to the above, confirm that for *Hormosira*, there are distinct mechanisms controlling the cytoplasmic K^+ and Na^+ levels (Bergquist 1958). For example, the Na^+ mechanism is susceptible to cyanide at various concentrations and pH's, to inhibition by 2,4-dinitrophenol and by the sulfhydryl-complexing agent, *p*-chloromercuribenzoate. It may be cytochrome mediated. On the other hand, the important feature of the K^+ regulating mechanism is its insensitivity to all inhibitors tested except 2,4-dinitrophenol.

It has also been shown that the cellular Na^+ level is below that of the surrounding sea water, and that the K^+ concentration is much higher — about 0.7 *M*. If we accept the existence of two separate mechanisms of cation regulation a possible explanation can be advanced. It can be expected that excess Na^+ in the surrounding medium will have little effect on the oxygen uptake, since Na^+ ions are continually diffusing into the cell and being 'pumped' out by the Na^+ mechanism, and it would appear very likely that the enzyme system responsible is working at full capacity. The K^+ ions are transported into the cytoplasm by some mechanism, the exact nature of which is not fully clear at present, perhaps via an autoxidisable cytochrome mechanism similar to that found in aroid spadix tissue (Bonner 1957), but it would appear likely that there is an increased oxygen uptake as energy is expended to accumulate the ions against their electrochemical potential gradient.

Convincing interpretation of the inhibitory effect of the chloride ion cannot be advanced at present. It was expected that it too would induce a respiratory stimulation if accumulation was effected by a Lundegårdh-type scheme (Lundegårdh 1954), but it would appear that such a system of anion transport is untenable for *Hormosira*, at least at high chloride concentrations. It must be remembered also that 'anion respiration' has mainly been demon-

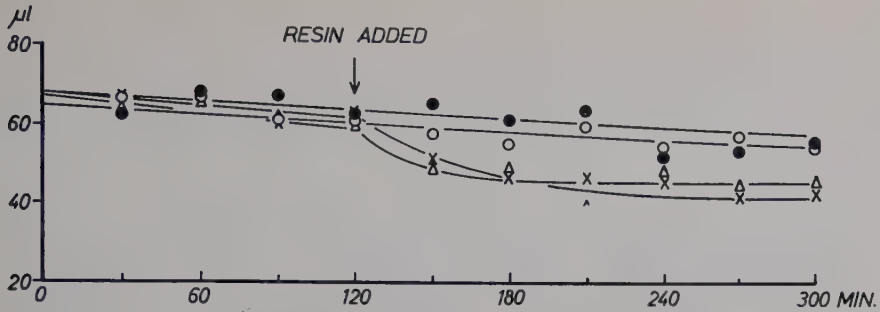


Figure 3. Effect on respiratory oxygen uptake of added Cl^- ions. Tris-buffered natural seawater, pH 9.4. Diacritic symbols: ○ — control, $\text{Cl}^- = 530$ $\mu\text{equiv./ml.}$, ● — IR-4B- OH^- added, $\text{Cl}^- = 530$ $\mu\text{equiv./ml.}$, △ — IR-4B- Cl^- added, $\text{Cl}^- = 670$ $\mu\text{equiv./ml.}$, × — IR-4B- Cl^- added, $\text{Cl}^- = 703$ $\mu\text{equiv./ml.}$ Ordinate as in Figure 1.

strated for higher plants, especially storage organs and roots (Robertson 1941, 1956, Epstein 1955), and to the writer's knowledge, has not been reported for marine algae. It is interesting to note that in an investigation of the uptake of radioiodine by *Hormosira*, it has been observed that there is no stimulation of respiration when the anion (radioactive plus carrier sodium iodide) is added to fresh tissue, and there is frequently a depression of oxygen uptake with well-washed tissue (Bergquist, unpublished).

Summary

1. There is a stimulation of respiration when tissue of the marine alga *Hormosira banksii* is exposed to K^+ ions adsorbed on a weakly acidic cation exchanger, Amberlite IRC-50.

2. There is no effect on the rate of oxygen uptake when Na^+ is used as the cation, and if Cl^- ions are added adsorbed on a weakly basic exchanger, there is a decrease in the oxygen consumption.

3. These results are taken as evidence supporting the suggestion that there are independent mechanisms for cation uptake in *Hormosira*. Explanation of the chloride effect cannot be advanced from the data available.

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Utilisation of Sucrose by Three Soft Rot Bacteria

By

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I. Introduction

In a previous paper, the authors (1954) reported that the three closely related soft rot bacteria, *Bacterium aroideae*, *B. carotovorum*, and *B. phytophthorum* readily hydrolyse sucrose in their culture media and utilise appreciable amounts of its hydrolytic products. Maltose, on the other hand, is utilised by these organisms to a very small extent when compared to sucrose. The ratio of direct to total reducing value of maltose which amounts to 50 % does not undergo any appreciable changes when maltose-containing media are inoculated with each of the three bacteria under test. The above results speak strongly for the fact that the enzyme concerned with sucrose hydrolysis by these organisms is not of the α -glucosidase type, lest it should have broken down maltose in a more or less similar way to sucrose. The fact that the three bacteria hydrolysed the fructoside raffinose in their culture media led the authors to support the view held by Saïd and his co-workers (1945, 1949, 1950, 1953 a, 1953 b) namely, that sucrose utilisation is effected through a process of hydrolytic cleavage by an enzyme of the fructofuranosidase type. It may be argued, however, that the fructofuranosidase enzyme demonstrated in the bacterial cells is responsible for the utilisation of the fructoside raffinose only while sucrose is still metabolised through the mediation of a specific sucrose phosphorylase enzyme (Mandels 1953, 1954), inspite of the presence of β -h-fructosidase enzyme in abundance to the metabolic requirements.

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The experiments reported in the present work were thus planned with a view to throwing some more light on the nature of the enzyme concerned with the utilisation of sucrose by the above mentioned bacteria.

II. Materials and Methods

The organisms used in this investigation were as follows:

1. *Bacterium aroideae*, (Townsend) Stapp; Dowson's strain, 66.
2. *Bacterium carotovorum*, (Jones) Lehmann and Neumann.
3. *Bacterium phytophthorum*, (Appel) Burgwitz.

The three cultures were kindly supplied by the Plant Bacteriological Laboratory of the Imperial College of Science and Technology, London.

In this series of experiments a basal medium of the following composition was prepared:

Asparagin	10 g.
MgSO ₄ · 7 H ₂ O	2.5 g.
KH ₂ PO ₄	5 g.
Distilled water to	500 ml.

The various sugars or mixtures of sugars tested were dissolved separately in sterile distilled water at double the required concentration. Equal volumes of the sugar solution and the basal medium were mixed. The full culture media so prepared were distributed into a series of sterile 150 ml. conical flasks at the rate of 10 ml. per flask and inoculated at the rate of 0.2 ml. of a suspension of each of the three bacteria under test in sterile distilled water. The flasks were then incubated at 25°C for the specified time intervals at the end of which sugar contents of the various media were determined. The methods used for sugar analysis of the media were those referred to by the authors in a previous paper (1954).

III. Experimental Results

1. Utilisation of sucrose and its constituent monosaccharides

The first experiment in this series was carried out to compare the rate of utilisation of sucrose with that of its constituent monosaccharides.

To the basal medium the following sugars or mixture of sugars were added to furnish the specified concentrations (‰):

Sucrose	2
Glucose	2
Fructose	2
Glucose 1+Fructose	1

The culture media so obtained were inoculated with each of the three test bacteria and incubated at 25°C for 48 hours at the end of which sugar contents of the various culture media were determined.

Table 1. Sugar uptake by *B. aroideae*, *B. carotovorum*, and *B. phytophthorum* in 48 hours from media containing sucrose, glucose, fructose or a mixture of glucose and fructose. (Calculated as mg. glucose per ml. medium.)

Organism	Sugar in culture medium %	Sugar uptake in 48 hours
<i>B. aroideae</i>	2 Sucrose	16.02
	2 Glucose	18.00
	2 Fructose	10.52
	1 Glucose +	Total 14.25
	1 Fructose	Glucose 7.33 Fructose 6.92
<i>B. carotovorum</i>	2 Sucrose	16.10
	2 Glucose	14.70
	2 Fructose	14.70
	1 Glucose +	Total 15.00
	1 Fructose	Glucose 7.27 Fructose 7.73
<i>B. phytophthorum</i>	2 Sucrose	8.55
	2 Glucose	8.25
	2 Fructose	2.33
	1 Glucose +	Total 4.50
	1 Fructose	Glucose 2.87 Fructose 1.63

The amounts of sugar absorbed by each of the three bacteria from the various culture media are calculated and presented in Table 1 which shows that:

1. The highest amount of sugar absorbed in 48 hours by *B. aroideae* was from glucose medium followed by sucrose, then the mixture of glucose and fructose; the lowest amount being absorbed from the fructose-containing medium. The uptake of glucose and fructose from their mixture was almost equal at the end of 48 hours.

2. The total amount of sugar absorbed by *B. carotovorum* from the different media was, more or less, the same. This organism absorbed almost equal or even slightly greater amounts of fructose than glucose from their mixture.

3. *B. phytophthorum* absorbed more sugar from sucrose- and glucose-media than from the other two media. The rate of absorption of fructose from its mixture with glucose was somewhat slower than that of the glucose component.

2. Effect of heating bacterial cells on their power of utilising sucrose

In this experiment the three bacteria were subcultured on sucrose culture medium, incubated for 24 hours and the crop of each bacterium collected at the end of this period, centrifuged and washed several times with sterile water. The bacterial cells

Table 2. Amounts of sucrose utilised by heated and unheated cells of *B. aroideae*, *B. carotovorum*, and *B. phytophthorum* in one hour. (Calculated as mg. sugar per ml. medium.)

Organism	Treatment of bacterial cells	Sucrose utilised
<i>B. aroideae</i>	Heated at 60°C for 10 minutes	0.71
	Unheated	1.35
<i>B. carotovorum</i>	Heated at 60°C for 10 minutes	0.71
	Unheated	1.18
<i>B. phytophthorum</i>	Heated at 60°C for 10 minutes	0.59
	Unheated	0.77

were then suspended into sterile water and divided into two portions. One portion was heated in a water bath at 60°C for 10 min.; the other was kept as control. This heat treatment was found by preliminary experiments to kill the bacterial cells but does not seriously affect the activity of invertase enzyme solutions. The heated and unheated bacterial cells were then inoculated into a 2 % sucrose solution and incubated at 25°C for one hour. Examination of the culture cycle of each of the three bacteria under test revealed that all of them were usually in the lag phase during the first hour after inoculation into their culture media. Therefore, no appreciable increment is to be expected in the amount of invertase enzyme in the unheated crop of each bacterium over the heated one during this short duration of the experiment. At the end of the experimental period the media were analysed for their contents of reducing sugars and the values of sucrose utilised (reducing sugar's appearing in the medium plus sugar uptake from the corresponding medium) in each case are calculated and presented in Table 2.

Table 2 shows that heated cells of the three bacteria utilised slightly smaller amounts of sucrose than the corresponding unheated ones. The small values recorded for sucrose utilisation are presumably due to the very short duration of the experiment. The slightly higher values for sugar utilisation by the heated over the unheated cells is, most probably, due to the inactivation of some of the invertase enzyme when heated to 60°C.

The above results support the view that sucrose is utilised by these organisms through the mediation of an enzyme of the fructofuranosidase type rather than a specific sucrose phosphorylase enzyme since the presence of the latter enzyme should have induced appreciable increase in the amounts of sucrose utilised by the unheated over the heated cells.

3. Effect of glucose and fructose on sucrose utilisation

This experiment was designed to study the effect of glucose and fructose on sucrose utilisation by the three test bacteria. For this sake culture media were prepared by adding the following sugar or mixtures of sugars to the basal medium to give the specified concentrations (%):

Sucrose	2
Sucrose 2+Glucose	2
Sucrose 2+Glucose	5
Sucrose 2+Fructose	2
Sucrose 2+Fructose	5

The various media were inoculated with each of the three bacteria, incubated at 25°C for 24 hours at the end of which the media were analysed for their direct and total reducing values.

From the results of the analysis of the various media the total sugar uptake by each of the three bacteria from its culture media is calculated and set out in Table 3 which shows that the total amounts of sugar taken up by each bacterium increased with the increase in concentration of sugar in all the experimental media except in case of *B. carotovorum* which absorbed less sugar from the medium containing 5 % glucose than from that with 2 % glucose. Yet, *B. carotovorum* absorbed more sugar from the above two media than from the control medium containing 2 % sucrose only. The above results refute the possibility of any toxic effect of the high concentrations of sugars used on the vital activities of the three bacteria.

The amounts of sucrose which disappeared from the various culture media being taken up by the bacteria or recovered in the media as hydrolysis products are calculated and presented in Table 4. The result which stands out clearly in Table 4 is the appreciable reduction in the amounts of sucrose which disappeared from the culture media of the three bacteria in presence of glucose or fructose, the reduction being more pronounced in presence of the former than the latter sugar.

Such reduction in the amounts of sucrose utilised by the three bacteria in the presence of glucose or fructose may be explicable on the basis of the availability of a ready-made hexose, glucose or fructose, which is quite suitable for the growth of the three bacteria. The greater retardation of sucrose

Table 3. Total sugar uptake by *B. aroideae*, *B. carotovorum*, and *B. phytophthorum* in 24 hours when inoculated into culture media containing sucrose or mixtures of sucrose and glucose or fructose. (Calculated as mg. sugar per ml. medium.)

Organism	Sugar or mixture of sugars in culture medium				
	2 % sucrose	2 % sucrose + 2 % glucose	2 % sucrose + 5 % glucose	2 % sucrose + 2 % fructose	2 % sucrose + 5 % fructose
<i>B. aroideae</i>	11.20	16.93	20.73	19.73	25.33
<i>B. carotovorum</i>	10.64	20.68	15.10	23.54	22.99
<i>B. phytophthorum</i>	4.48	8.73	11.74	9.56	14.80

Table 4. Amounts of sucrose utilised by *B. aroideae*, *B. carotovorum*, and *B. phytophthorum* when inoculated for a period of 24 hours into culture media containing sucrose or a mixture of sucrose and glucose or fructose. (Calculated as mg. sugar per ml. medium.)

Organism	Sugar or mixture of sugars in culture medium				
	2 % sucrose	2 % sucrose + 2 % glucose	2 % sucrose + 5 % glucose	2 % sucrose + 2 % fructose	2 % sucrose + 5 % fructose
<i>B. aroideae</i>	15.92	4.36	0.67	8.29	10.08
<i>B. carotovorum</i>	10.64	9.29	4.48	11.20	7.84
<i>B. phytophthorum</i>	6.72	2.04	1.12	4.48	5.60

utilisation in presence of glucose than in presence of fructose is, most probably, due to varied degrees of the preferential absorption of the two hexoses from their mixtures with the disaccharide sucrose.

It may be mentioned, however, that the amounts of sucrose utilised by *B. aroideae* and *B. phytophthorum* (Table 4) increased with the increase in concentration of fructose in the medium; the reverse statement holds true for *B. carotovorum*. On the other hand, the increase in glucose concentration in the medium was accompanied by a marked reduction in the amounts of sucrose utilised by the three bacteria. The above results may be explicable on the basis of the assumption that the increase in concentration of fructose in the culture medium intensified the utilisation of sucrose by *B. aroideae* and *B. phytophthorum*, the latter sugar being much more preferably taken up by these two organisms than the former when each sugar is present singly in the culture medium (Table 1). Similar observations were recorded earlier by Saïd and Fawzy (1949 b) while feeding radish and carrot root slices with glucose and galactose either singly or in mixture when they found that the presence of galactose with glucose in the medium intensified the use of the latter sugar. This effect of fructose on sucrose utilisation was not observed in case of *B. carotovorum* presumably on account of the fact that the two sugars were almost equally utilisable by this organism. The observed reduction in the amounts of sucrose utilised by each of the three bacteria with the increase in concentration of glucose in the culture medium may be taken as an indication of the preferential absorption of the latter sugar from its mixture with sucrose.

4. Constitutive nature of the enzyme concerned with sucrose hydrolysis

After studying the enzymatic make-up of certain bacteria which had been grown on a variety of media, Karström (1930) concluded that bacterial enzymes could be divided into two general types:

a. Constitutive enzymes: which are always formed by a given organism and are independent of the composition of the medium on which it grows.

b. Adaptive enzymes: which are formed by a given organism only when growth takes place in presence of the specific substrate *i.e.* formed only when required.

An experiment was planned to test whether the enzyme concerned with sucrose utilisation by the three bacteria was constitutive or adaptive. For this sake the three bacteria were successively subcultured for 7 days, at 24 hourly intervals, into the basal medium to which sucrose, lactose or maltose were added to give a concentration of 2 % in each case. The crops obtained at the end of each 24 hour period were inoculated into a fresh sucrose culture medium and then incubated at 25°C for 24 hours at the end of which the culture media were analysed for their contents of reducing sugars.

The results obtained from this experiment revealed that the absence of the substrate (sucrose) from the culture medium for a period of 7 days did not induce any appreciable effect on the rate of utilisation of sucrose by *B. aroidae* and *B. phytophthorum*.

Analysis of culture media inoculated with *B. carotovorum* initially subcultured on lactose or maltose failed to reveal the presence of any reducing sugars, thus behaving in exactly the same way as when initially subcultured on sucrose culture media. Examination of all culture media for the presence of reducing sugars at the early stages of growth of this organism revealed the presence of measurable amounts of reducing sugars in all its culture media during the first 12 hours of incubation. These results show that continued subculturing of *B. carotovorum* on media free from sucrose did not alter its peculiar behaviour with regards sucrose hydrolysis to any appreciable extent.

The foregoing discussion of the results indicates that the three bacteria under test utilise sucrose through the mediation of a constitutive enzyme.

IV. Discussion

It has long been recorded that when sucrose was supplied in culture media of plant tissues it was always broken down at the protoplasmic surfaces of the plant cells and some of the products of sucrose breakdown were subsequently absorbed (Robbins 1918, Gawadi 1935, Saïd and his co-workers 1945, 1949 a, 1950, 1953 a, 1953 b, Burström 1941, Dimond and Peltier 1945, Dormer and Street 1949, Street and Lowe 1950, Tolba and Ghanem 1954, Harley and Smith 1956, and various other investigators). Owing to the fact that the rate of sugar uptake is always less than the rate of sucrose break-

down in the external media, reducing sugars usually appear in these sucrose media.

Gawādi (1935), Saïd (1937, 1941), and Burström (1941) suggested that sucrose breakdown in the culture media of plant tissues was brought about by means of invertase enzyme centres situated at the protoplasmic surfaces of the plant tissues. The evidence that enzymes are at cell surfaces has recently been a subject of review by Rothstein (1954). Saïd and his co-workers suggested that these invertase enzyme centres were of the fructofuranosidase type.

On the other hand, Dormer and Street and Street and Lowe postulated that sucrose breakdown in the media of their excised plant roots was by means of specific sucrose phosphorylase enzyme. This phosphorylase enzyme has been found in bacterial sources by Doudoroff (1943) and Doudoroff *et al.* (1943) and evidence has been given later by Doudoroff (1951) for the utilisation of intact sucrose by *Pseudomonas* species. Mandels (1953, 1954) claims that his results indicate that sucrose is metabolised by a non-hydrolytic system in spores of the fungus *Myrothecium verrucaria*. He stated that "the evidence available is consistent with the postulate that sucrose utilisation is mediated by a sucrose phosphorylase although attempts to demonstrate such an enzyme were unsuccessful".

Gottschalk (1949) refuted the direct schemes proposed by others for the fermentation of sucrose, maltose and lactose by yeast and concluded that there is no evidence contradicting a preliminary hydrolytic step.

The results of the present investigation showed that the three bacteria under test absorbed almost equal amounts of sucrose and glucose when these two sugars were present singly in their culture media. If a specific sucrose phosphorylase is present in the bacterial cells they would have been capable of using inorganic phosphate directly to form hexosephosphates from sucrose rather than making use of high energy phosphate bonds to phosphorylate the hexose and hence one would expect a much higher uptake from sucrose than from glucose (as in case of spores of *Myrothecium verrucaria*; Mandels 1953, 1954). Such result was not reached in the present experiments, a fact which indicates that sucrose is utilised by these organisms through a process of hydrolysis by an enzyme of the fructofuranosidase type, the presence of which in the cells of the test bacteria has already been established (Tolba and Ghanem 1954), rather than through the mediation of a specific sucrose phosphorylase.

As a further proof to the above hypothesis, a comparison was made between the rate of utilisation of sucrose by normal bacterial cells and by cells heated at 60°C for 10 minutes. The results obtained showed that heated cells of the three bacteria utilised slightly smaller amounts of sucrose than the un-

heated ones. This result, though not very conclusive owing to the small amounts of sucrose utilised in the very short duration of the experiment, suggests that sucrose is utilised through the mediation of an enzyme of the fructofuranosidase type rather than a specific sucrose phosphorylase enzyme since the presence of the latter enzyme should have induced appreciable increase in the amount of sucrose broken down by the unheated over the heated cells. The slightly higher values for sucrose utilisation by the formers over the latter is, most probably, due to the inactivation of some of the invertase enzyme when heated at 60°C.

Examination of the results of the utilisation of sucrose by the three bacteria when this sugar was present in mixture with glucose or fructose revealed an appreciable reduction in the amounts of sucrose which disappeared from the media in presence of the hexoses than in their absence. This reduction is, presumably, due to the preferential absorption of the hexoses from their mixtures with sucrose. The increased reduction in sucrose utilisation with the increase in concentration of glucose may be taken as a circumstantial evidence supporting this view.

It may be mentioned in this respect that glucose induced greater retardation of sucrose utilisation by the test bacteria than fructose. Such varied effect of the two hexoses is, most probably, due to varied degrees of the preferential absorption of the two hexoses from their mixtures with sucrose.

From the foregoing discussion of the results it may be concluded that sucrose is utilised by the three bacteria under test through a process of hydrolytic cleavage effected by an enzyme of the fructofuranosidase type and not through the mediation of a specific sucrose phosphorylase enzyme. This conclusion verifies the previous findings of Tolba and Ghanem (1954) and is in complete agreement with those reached earlier by Saïd and his co-workers (l.c.) with regards sucrose utilisation by radish and carrot root slices as well as by mycelial mats of *Fusarium moniliforme*.

The continued daily subculture of the three test bacteria for a period of 7 days in presence and absence of sucrose did not induce any appreciable effect on the rate of hydrolysis of sucrose by the three bacteria, a fact which indicates that the three bacterial organisms utilise sucrose through the mediation of constitutive enzymes.

V. Summary

The possible enzymatic pathways of the utilisation of the disaccharide sucrose by the closely related soft rot bacteria, *Bacterium aroideae*, *Bacterium phytophthorum* and *Bacterium carotovorum* were investigated. The results obtained from these investigations led to the conclusion that sucrose utilisation

tion by the three bacteria takes place through the mediation of a constitutive invertase enzyme of the fructofuranosidase type and not through the action of a specific sucrose phosphorylase enzyme.

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The Role of Auxins in the Sex Expression of the Cucumber

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The induced modification of sex expression has been the subject of research of numerous workers ever since the early observations of Heyer (7) on the change in sex expression in monoecious plants by environmental factors (11, 14, 16 and others).

Reviewing the literature concerning the modification of sex expression, Heslop-Harrison (5, 6) concluded that sex expression in plants is regulated by the level of growth substances and that there are two different optima for the formation of male and female flowers respectively, the optimum for the female flowers being higher. These conclusions were derived mainly from growth substances treatment of plants, and not from an investigation of the level of natural growth substances. When the present investigation into sex determination of cucumber was initiated in 1954, it appeared to us that for a better understanding of sex determination in plants, it may be desirable to trace the changes in natural growth substances which accompany the change in sex expression. This can be accomplished in two ways: firstly, by subjecting plants to treatments which are known to influence the level of growth substances directly, and recording the resulting changes in sex expression; secondly, differences in the level of growth substances in lines of different sex expression may be compared (in various plant organs). Actually both methods were employed concurrently. It shall be noted, that for the second method, it is necessary first to establish lines which differ markedly in sex expression, yet are of similar pedigree. Such lines have been obtained in the course of genetic research, continued currently with these physiological investigations.

Table 1. *Measurements of sex expression in monoecious plants* (variety "Yorkstate Pickling" — selected monoecious line) *under natural* (13 hours) *and augmented* (18 hours) *photoperiods*.

Sex expression	Natural day length	Prolonged day length	Difference	p value of the difference
Number of staminate flowers per plant	115.7	108.2	7.5	0.05
Number of pistillate flowers per plant ...	2.0	1.0	1.0	0.01
Pistillate flowers				
Staminate flowers	0.0171	0.0093		
"Node number" (from the first node to first pistillate flower)	13.54	25.55	12.01	0.01

Materials and Methods

Plant Material. — In most trials, monoecious and female (gynoecious) lines of the pickling variety Yorkstate Pickling (YSP) were used. The gynoecious lines were obtained by repeated back-crossing (at least 5 times) to the ordinary monoecious type of the variety. The monoecious line of the variety used in this study, as well as those of other varieties, have been inbred for several generations.

Gynoecious plants differ conspicuously from monoecious ones, as they bear pistillate flowers only, except for the first new nodes of the main branch. The monoecious plants were described in detail in a previous publication (Shifriss and Galun 1956). A clearer picture of the lines used in this investigation may be obtained from Tables 1 and 2, which contain data on the sex expression of monoecious and (heterozygous) gynoecious plants grown at a natural 13-hours day and 18-hours day. Both in monoecious and in gynoecious plants, a considerable influence of day length has been found, such as has also been described (even though in monoecious plants only) in the work of Nitsch *et al.* (1952).

Measurements of sex expression. — After it had been found out in this laboratory (20), that node number is a reliable and simple measure of sex expression, we recorded the influence of the various treatments on sex expression in terms of *node number*. This term refers to the number of nodes from the base of the plant to the location of the first pistillate flower (on the main branch).

Analysis of growth substances. — After several preliminary trials based on the extensive literature available in this field (8, 13, 23, 24 and others), the following extraction method was used. Plant material was collected at a fixed time and dried by lyophilisation and mashed to powder. For extraction, the equivalent of 25 gr. of fresh leaf material were used. The material was extracted twice, with peroxide-free ether at 2°–4°C, evaporated under low vacuum and redissolved in lcc. of absolute ethyl alcohol.

Chromatographic separation was done by descending method, the solvent being isopropanol: ammonia: H₂O in a ratio of 80 : 5 : 15. After separation the paper was divided obliquely into 10 equal parts between the start line and the end line.

The method employed in the bio-assay was similar to that described by Nitsch and

Table 2. *Measurements of sex expression in gynoeocious plants (Variety "Yorkstate Pickling" — selected gynoeocious line) under natural (13 hours) and augmented (18 hours) photoperiod.*

Sex expression	Natural day length	Prolonged day length	Difference	p value of the difference
Number of staminate flowers per plant	2.33	13.5	11.17	0.05
Number of pistillate flowers per plant ...	13.33	9.08	4.25	0.01
Pistillate flowers	5.71	0.67		
Staminate flowers				
"Node number"	1.77	4.16	2.39	0.01
Number of nodes from basis to continuous female phase	4.00	8.91	4.91	0.01

Nitsch (1956), but length of coleoptile sections was 6.5 mm. and red light was given at the second day of germination.

Other methods and details of methods differing from the above, will be found with the records of the respective experiments.

Results

1. *The effect of removal of leaves*

It is generally agreed that leaves play an important part in the synthesis of growth substances, and that in young leaves this synthesis proceeds at a higher rate than in mature ones. Therefore, any artificially induced change in the ratio of young to mature leaves, may cause a change in the balance of growth substances. If there is any relationship between growth substances balance and sex expression, a change in the ratio young to adult leaves must cause a change in the sex expression, too. But since growth substances function first and foremost in elongation, any change in sex expression must be accompanied by changes in elongation. The following experiments are based on these arguments.

Effects of removal of young leaves only. — In this experiments we proceeded from a somewhat "reversed" point of view. The basic assumption was here that if monoecious and gynoeocious plants did not differ in growth substances balance, changes in elongation brought about by removal of young leaves would be similar in both types. However, if a difference in growth response to removal of young leaves should be found, we would have to conclude that the difference in sex expression was connected with natural growth substances.

A line segregating approximately in a 1 : 1 ratio into monoecious and gynoeocious plants of the variety YSP was used. In half the number of plants, leaves

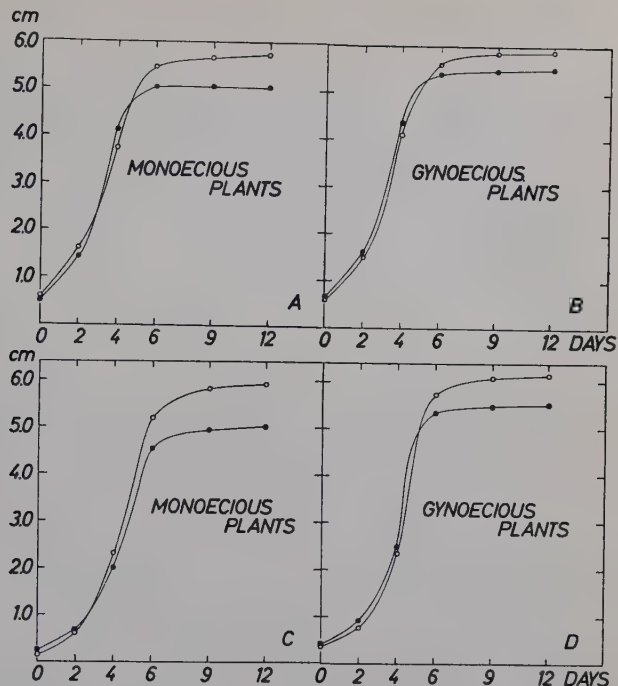


Figure 1. — Effect of removal of young leaves on the growth of the second (A, B) and third (C, D) internodes: ○ — leaves intact, ● — leaves removed.

were removed simultaneously from the second and third nodes. This was done when the younger leaf measured approximately 2 cm. along the main leaftrace, and older one had reached about its final size. The second group of plants was left intact. Measurements were recorded before the sex of the plants has been observed; data were afterwards grouped according to sex and to treatment. This procedure insured unbiased results.

The data have been summarized in the form of a growth curve in Figure 1, in which the effect of leaf removal on the elongation of the second and third internode (above the removed leaves) in monoecious and gynoeceous plants were followed for 15 days after removal. It appeared in all four comparisons that in plants from which leaves have been removed, the elongation of the internodes below the point of removal is inhibited.

In Table 3 the final length of the second and third internodes in both plant types has been recorded. It is apparent from the data that in both comparisons (2nd and 3rd node) the difference between *treated* and *untreated* plants in node length is greater in the monoecious than in the gynoeceous group. The difference in length of the second internode among the monoecious plants caused by the removal of leaves is 0.70 cm., $t=2.8$ (significant), while the difference among the gynoeceous plants, caused by the same treat-

Table 3. *The effect of removal of young leaves on the elongation of adjacent internodes in monoecious and gynoeceious cucumber plants.*

Sex of plants	Treatment (young leaves removed or not removed)	No. of plants	Length in cm.	
			Second internode	Third internode
Monoecious	Control	23	5.7	5.9
Monoecious	Leaves removed	21	5.0	5.0
Gynoeceious	Control	25	5.9	6.2
Gynoeceious	Leaves removed	25	5.5	5.5
LSD	0.01		0.7	0.6
LSD	0.05		0.5	0.4

ment, in only 0.4 cm., $t=1.5$, and is non-significant. A similar difference was found for the third internode. It may be concluded from this experiment that removal of leaves causes an inhibition of elongation in both sex types, but that the effect of the treatment is more pronounced in monoecious plants. On the other hand, the difference in internode length between untreated plants of different sex types was found non-significant.

Whatever the explanation to those results may be, the data seem to indicate that difference in sex expression, must be looked for not only in the young leaves but also in the mature leaves, and perhaps primarily there.

Effect of removal of young and mature leaves. — The following experiment has been based on the conclusion just described. It dealt with the influence of leaves of various ages on the development of sex expression (determined by "node number") and on elongation; in the main stem and in the side branches.

A monoecious line of the local variety "Beith-Alpha" was used. Four treatments were included in the experiment, as follows: — 1) Control (no removal of leaves); 2) continuous removal of every other leaf at the time of its separation from the growing point; 3) continuous removal of every other leaf as soon as it attained full size; 4) continuous removal of every leaf as soon as it attained full size.

Measurements which included plant length, number of leaves developing on the plant (including removed leaves), length of side branches, and "node number", were taken approximately one month after planting (Table 4).

It may be noted that removal of young leaves has a very marked effect on elongation. The average height of plants from which such leaves had been removed was only 24.1 cm., as compared with 38.3 cm. of control plants. Removal of every other, or all mature leaves, did not cause any significant change in plant height. This may be explained by the lack of assimilates in these plants.

In spite of the difference in total height, no difference in number of leaves

Table 4. *The effect of removal of young and adult leaves on the development and sex expression of monoecious cucumber plants.*

Treatment	No. of plants	Length of main branch (in cm.)	No. of leaves unfolding on main stem	Length of side branch developing from third node	"Node number"
I Control	15	38.3	12.4	4.4	16.3 ¹
II Every other leaf removed while young	11	24.1	11.0	11.0	19.2 ¹
III Every other leaf removed ²	14	38.9	12.4	5.6	14.1 ¹
IV Every leaf removed ²	17	34.7	11.5	2.7	14.1 ¹
LSD 1 %		8.3	1.9	8.7	
LSD 5 %		6.0	1.4	6.3	

¹ All differences between treatment except the difference between III and IV, are highly significant (at the 1 % level).

² After reaching full size.

developing subsequent to the various treatments were found, *i.e.* the internodes in treatment II were on the average shorter than in the other treatments. It appears that the strongest branching tendency was observed in plants of treatment II — removal of every other "young leaf". In those plants in which all mature leaves were removed, branching was least pronounced.

The strongest female tendency (lowest node number) was observed in treatments III and IV, in which mature leaves were removed. Treatment II, in which young leaves were removed, caused the highest node number.

Altogether, it was shown that the removal of young leaves, which apparently produce large quantities of growth substances, results in the formation of a high node number, while on the other hand, the removal of mature leaves results in a stronger female tendency than the untreated plants. It was also found that with development of the male tendency resulting from treatment II, elongation was inhibited as expected.

Leaf removal combined with application of growth substances and inhibitors. — A monoecious line of the variety "Beith-Alpha" was used. Seeds were planted in pots in the greenhouse. The experiment included 5 treatments: 1) control, 2) removal of every other young leaf, 3) removal of every other young leaf and application of 0.1 % NAA in lanolin ointment to the cut surface of the petiole, 4) removal of every other mature leaf, 5) removal of every other mature leaf and application of coumarin in lanolin ointment to the cut surface. Results were summarized in Table 5 (all differences between treatments except the difference between 4 and 5 are significant).

It appears that the removal of young leaves diminishes the female tendency, while the removal of mature leaves diminishes the male tendency —

Table 5. *The effect of removal of young and adult leaves and treatments with growth promoting and growth inhibiting substances.*

Serial No.	Treatment	No. of plants	"Node number"
1	Control	21	6.8 ± 0.4
2	Every other young leaf removed	18	11.6 ± 0.7
3	Every other young leaf removed and cut surface treated with NAA	18	5.2 ± 0.6
4	Every other adult leaf removed	22	8.2 ± 0.4
5	Every other adult leaf removed and cut surface treated with coumarin	20	8.2 ± 0.2

node number is higher than in the controls (a verification of results of the previous experiment). The addition of NAA to plants from which the young leaves have been removed, results in a node number which is even lower than in the control, but the addition of coumarin to plants from which mature leaves were removed did not affect the node number, which remained similar to node number in treatment IV. The added 0.1 % NAA apparently has a stronger effect than the natural growth substances produced in young leaves, which were removed from the plants. The fact that it is possible through removal of young leaves to modify sex expression towards the male side and by application of synthetic growth substances to shift it back towards the female side, showed that the effect of young leaves on sex expression may be mediated through the control of the supply of growth substances.

A parallel effect of inhibitors could not be determined but this does not prove that naturally occurring inhibitors have no effect, as it is quite possible that the choice of coumarin was not very fortunate, or that its concentration was unsuitable.

2. *Determination of growth substances and inhibitors in growing points and leaves of monoecious and gynoeceious plants in various development stages*

The limit of sensitivity of the present method was first tested for IAA and indoleacetonitrile (IAN) solutions (Figure 2).

At first, mature leaves — *i.e.* leaves removed for analysis when they had reached full size, and before any symptoms of senescence appeared — of monoecious and gynoeceious lines were analysed. Analysis of both sex types was always made in the same day, in order to afford a better comparison.

The result of three such analyses, which give a reliable representation of other similar samples, are summarized in Figure 3. The striking difference between sex types with regard to the inhibition zone between Rf. 0.0. and 0.3 is demonstrated most clearly by these data. Stimulation zones, between Rf.

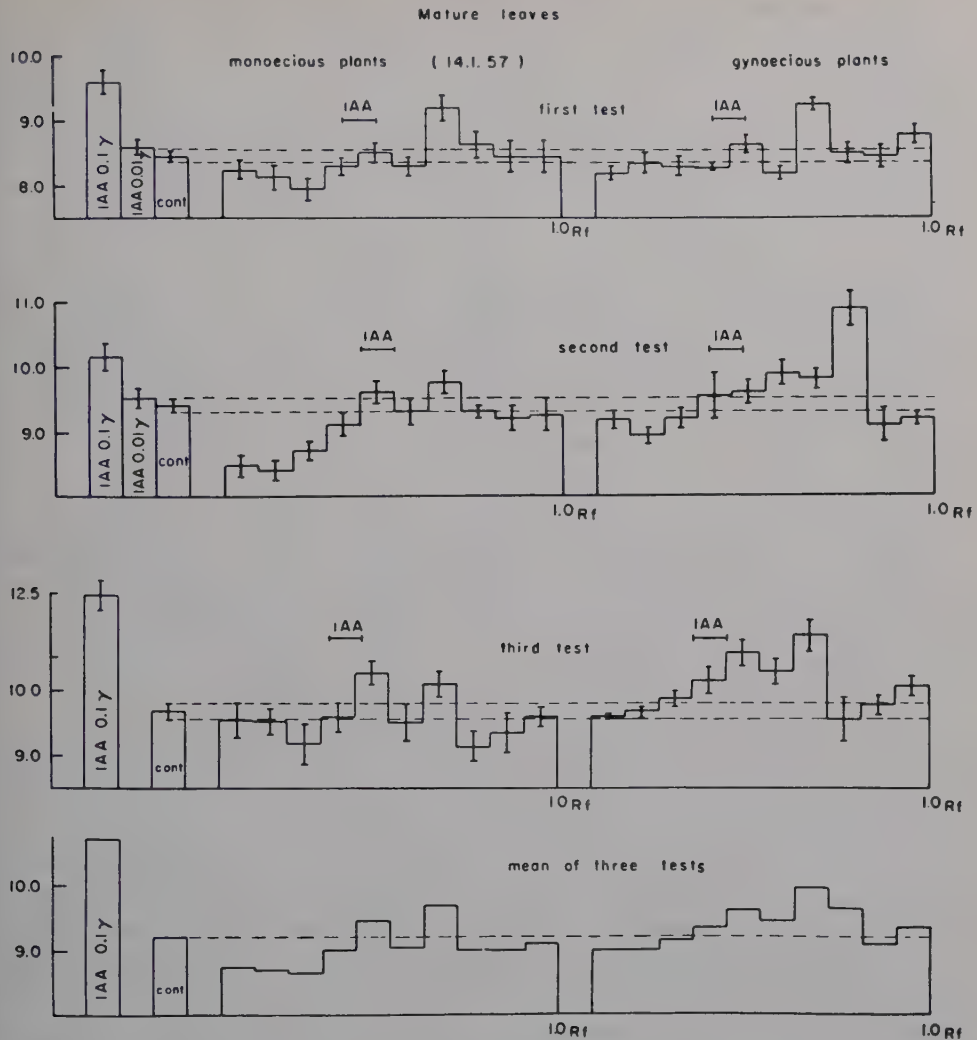


Figure 2. — Histograms of *Avena* coleoptile sections grown in various concentrations of IAA and IAN. The height of the columns represent mean length in mm. after incubation; the reaction to each concentration was tested twice. Confidence intervals are marked at the top of each column.

0.4—0.5 and Rf. 0.6—0.7 are similar in monoecious and gynoeceous plants, and difference between sex types in the level of stimulating substances are not at all significant.

Some typical analyses of young leaves are summarized in Figure 4. For this test, 2 young leaves were removed from each plant. In these plants, too, in-

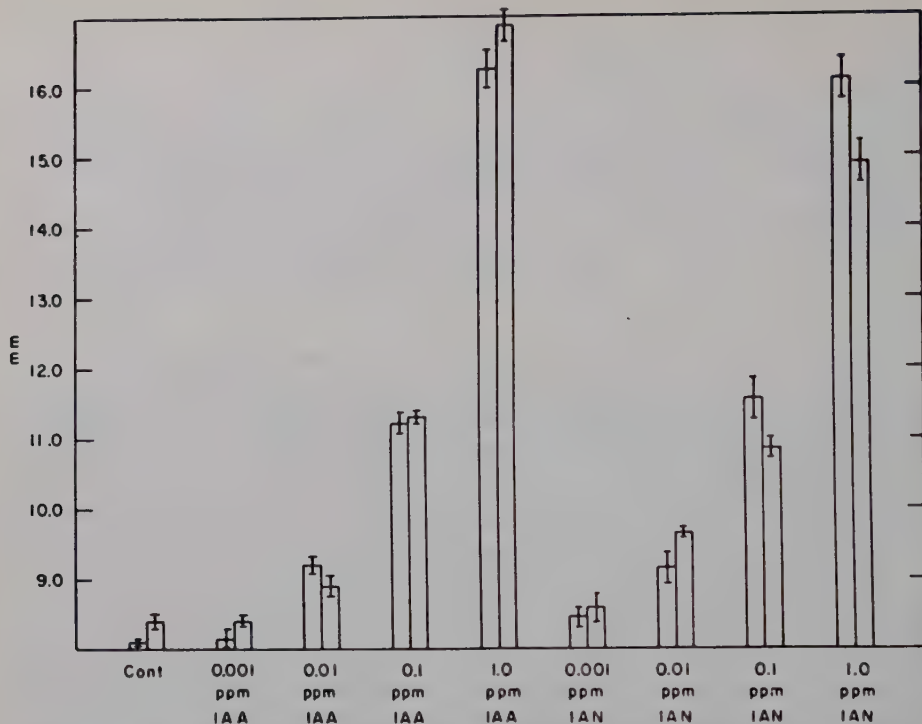


Figure 3. — *Histograms of bioassays of ether extractions from mature leaves.* The test of leaves from monoecious and gynoecious plants were made simultaneously. Other details as in Figure 3.

hibition zone was found between Rf. 0.0—0.3, but the sex type did not differ much with regards to this zone, and the inhibition itself was less marked than in mature leaves. On the other hand, the two stimulation zones were more conspicuous than in mature leaves.

When apices are examined, even more striking results are obtained (Figure 5). Firstly, there is hardly any inhibition zone; secondly, an almost continuous stimulation zone extends between Rf. 0.3—0.8. It is difficult to determine whether this is due to the appearance of a new stimulation zone between those found previously in young and mature leaves, or to an extension of the old zone. It would be difficult to identify a separate zone with the present methods, even though the chromatographic sheet in tests, the data of which is not present here, was divided into a large number of sections.

Comparison of the level of inhibiting substances in mature leaves of gynoecious and monoecious plants. — Since previous results indicate that the most pronounced differences between sex types was to be found in the inhibition

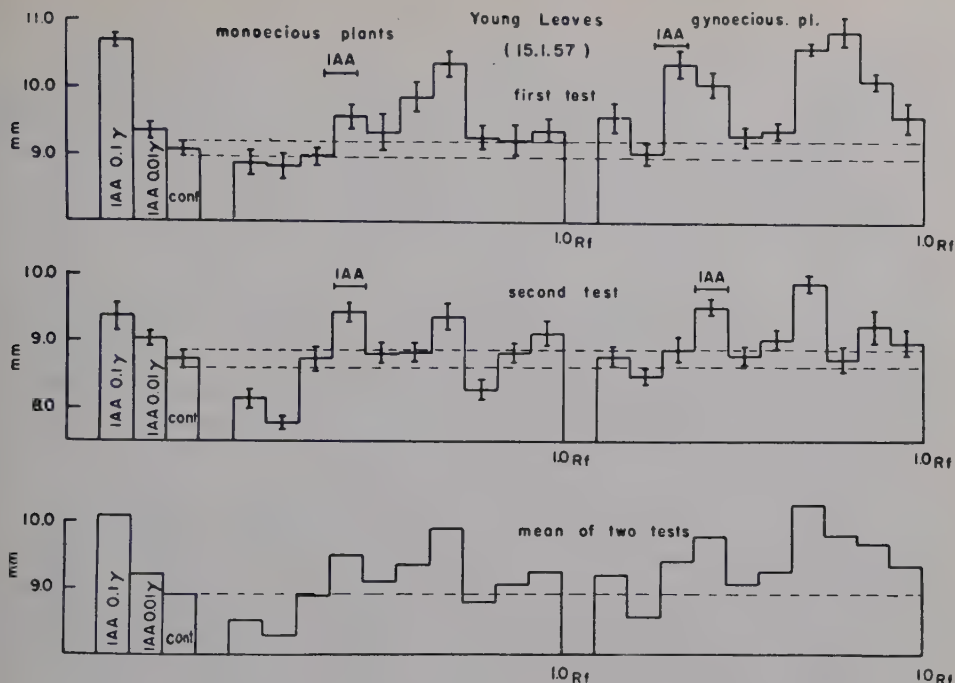


Figure 4. — Histograms of bioassays of ether extractions from young leaves.

zone, of mature leaves, another experiment was carried out, in which only those sections of the chromatographic paper between Rf. 0.0—0.3 were tested. Three such samples were taken from gynoeious and three from monoecious plants. Since this experiment dealt with inhibition only, wheat coleoptiles were used for these tests. Thirty coleoptiles were used for each section, *i.e.* 90 coleoptiles were used to test leaf exhaust from monoecious plants, and 90 — from gynoeious plants. Data are summarized in Table 6. Although the test which applied to the data of the entire zone (Rf. 0.0—0.3) proved that the difference between sex types was highly significant, the source for the differences in this test is only Rf. 0—0.2. Thus, results of the previous experiments were confirmed in a quantitative way.

Presence of IAA in cucumber leaves. — In order to determine whether the stimulation zone near Rf. 0.35 contained IAA, several preliminary experiments were made, as a result of which the following methods were subsequently used: apices of monoecious plants (approx. 10 g.) and of gynoeious plants were extracted with ether. After drying, the residue was dissolved in ethanol. The entire alcoholic solution of each sex type was spread along the starting line of the chromatographic paper. After the first separation and

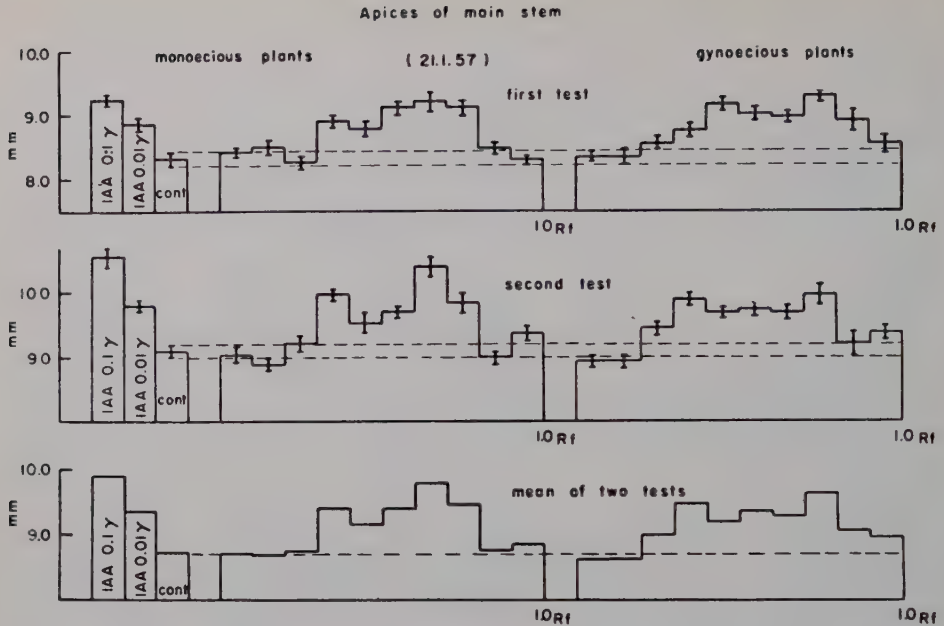


Figure 5. — Histograms of bioassays of ether extractions from stem apices.

drying, the region between Rf. 0.25—0.55 was extracted again, the volume of the ether was decreased over partial vacuum, and the entire ether solution was again separated by paper chromatography. The spraying with dimethylaminobenzaldehyde revealed spots which amounted to approximately 0.1—0.2 micrograms, but quantitative differences between monoecious and gynoeceous plants were difficult to determine. Therefore, IAA may be expected to be one of the natural growth substances of the cucumber plant; the lack of a marked quantitative difference in IAA of apices between the sex types agreed with the results of the bio-assay.

Table 6. *The effect of sections 1—3 (Rf. 0.0—0.3) from paper chromatography of adult leaves-extract of monoecious and gynoeceous cucumber plants, on the growth of wheat coleoptile sections.*

Rf.	Length of coleoptile section (mm.)	
	extract of monoecious plant	extract of gynoeceous plant
0.0—0.1	10.7 ± 0.1	11.10 ± 0.11
0.1—0.2	10.5 ± 0.2	11.27 ± 0.18
0.2—0.3	10.9 ± 0.2	11.09 ± 0.13

Discussion

As described in a previous paper (Shifriss and Galun 1956) three phases may be discriminated in cucumber plants: The staminate, the monoecious (mixed) and the pistillate phase; node number, which in this case can serve as a measure of sex expression, defines the point of transmission from the first to the second phase. The node on the main branch at which an uninterrupted sequence of pistillate flowers begins, marks the transmission from the second to the third phase. We may observe in cucumber plants, a gradual transition from the staminate phase through the mixed to the pistillate phase. Genetic and non-genetic factors may shift the starting points of the phases and by that change the sex expression. Our results should be interpreted thus: all treatments mentioned here and in our previous publication (Galun 1956), which brought about changes in node number, actually changed the location of the various flowering phases of the cucumber plant.

The influence of leaf removal at a different development stage was investigated by Barlow and Hancock (1956), but this work was not concerned with effect of the treatment in sex expression, but with the effect of leaf removal on internode length and leaf area in apple rootstocks. Our leaf removal experiments indicated a relationship between growth substances, internode elongation, ratio of adult to young leaves, and sex expression. On one hand, the two sex types differed in reaction to the same treatment — removal of young leaves — monoecious plants were more strongly effected than gynoeceious ones, and the difference in reaction also included a charcter dependent on growth substances — internode elongation. On the other hand, the reaction of plants of the same sex (monoecious) to different treatments revealed that while the store of natural growth substances was exhausted and elongation was inhibited, the first, staminate, phase lasted for a longer period of time (node number measured) and the appearance of later phases was delayed.

The results of the analysis of growth substances and inhibitors in cucumber leaves solve the question regarding the function of leaves in the development of sex expression and complements the results of the chemical vernalization (Galun 1956) and leaf removal experiments; it was found that in adult leaves of monoecious plants, inhibitors are more abundant than in adult leaves of gynoeceious plants.

Summary

In order to investigate the relationship between growth substances and sex expression, several experiments were conducted with monoecious and gynoeceious plants. It was the purpose of these experiments to determine differences

in the amount of growth substances and inhibitors which might be correlated with changes in sex expression.

It was found that the removal of young leaves from monoecious plants inhibit elongation more than removal of such leaves from gynoeceious plants. It was also found that removal of young leaves promote the male tendency, inhibits elongation, and causes strong branching, while the removal of mature leaves has the opposite effects.

Measurements of growth substances and inhibitors in extracts of stem apices, young and mature leaves, chromatographic separation and the *Avena* coleoptiles tests showed that monoecious and gynoeceious plants differ mainly in the nature of an inhibition zone in the chromatograms of mature leaves. This zone is more prominent in the chromatograms of monoecious plants than in that of gynoeceious plants.

As there seem to exist three phases in the flowering sequence of the cucumber plant — male (staminate flowers), mixed (staminate and pistillate flowers) and female (pistillate flowers), any treatment or any genetic character which brings the mixed and the female phase closer to the base of the plant, causes a more female sex expression, while any treatment which prolongs the duration of the first phase, delays the appearance of the second phase or suppressing it altogether, results in male sex expression.

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Description and Physiological Properties of Lipid-Containing, Non-Chlorophyllous Cells in Elodea

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1. Introduction

Although *Elodea* has been extensively used in nearly every phase of botanical study, apparently no information has been published concerning the presence of idioblastic lipid cells in the leaves of this plant. This is doubly surprising since these cells are so conspicuous and since the plant is such a popular one for research purposes.

It is the purpose of this paper to describe briefly the morphological appearance of these cells, their responses to and affinity for various non-vital stains and finally, some of their physiological properties, such as resistance to plasmolysis, ability to accept vital stains, pH of cell vacuole and occurrence of some enzymes.

The work was done with *Elodea densa* from two sources and *E. canadensis*. *E. Nuttallii* was not investigated. Preliminary examinations clearly indicated that in *E. densa* some sort of fatty substance was present in large amounts, so, for convenience, these idioblastic cells shall be referred to hereafter as "fat cells". The fat cells do not occur in *E. canadensis*.

2. Methods

No sectioning was required since the leaves of *E. densa* are only two to three cells thick. Entire, untreated leaves were examined under bright field and phase contrast.

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Several tests were made to determine the presence of proteins, carbohydrates, tannins and lipids in the fat cells. Tests for proteins included: a) the ferricyanide reaction of Hartig-Zacharias (Gomori). Fresh leaves were treated for 10 minutes in a 5 % solution of potassium ferricyanide in dilute hydrochloric acid. The leaves were then washed and flooded with a dilute solution of ferric chloride. Proteins stain blue. b) Tannin-ferric method of Salazar (Gomori). Leaves were treated about fifteen minutes in 5 % tannic acid in 5 % acetic acid, washed and flooded with ferric chloride. Proteins stain black. Leaves were also stained with aceto-carmine and with Buffalo Black in an attempt to stain nuclei in the fat cells.

There are no direct tests for sugars and indirect methods are employed. In this instance, leaves were defatted in ethyl alcohol, ethyl ether, or acetone for 24 hours, washed and then treated with Schiff reagent, omitting pretreatment with periodic acid. Positive reactions may be taken to indicate the presence of carbohydrates (Lillie). Starch was tested for by flooding the leaves with iodine dissolved in one molar potassium iodide.

Included among the tests for tannins were: precipitation from solution by potassium dichromate, a black or blue-green color with ferric salts, precipitation from solution by metal salts such as the chlorides of lead and copper, and a deep red color with potassium ferricyanide. None of these tests are specific for tannins but all true tannins answer them as a whole (Haas and Hill).

A number of dyes and stains were used to demonstrate the presence of lipids. Sudan III, Sudan IV, and Sudan Black B were used in 1 % concentrations or saturated solutions in distilled water, in ethyl alcohol and in ethylene glycol. Ethylene glycol consistently gave better results. 0.2 % Nile Blue A in aqueous solutions was used as an indicator for fatty acids. Leaves were also placed in 2.0 % osmium tetroxide for 15 to 20 minutes. Although this reagent has lost most of its prestige as a fat indicator, it is still valuable in a general way to indicate unsaturation.

In an attempt to demonstrate the presence of a vacuole in the fat cells, leaves were placed in dilute solutions (1/10 T) of Neutral Red at pH 7.0 for 24 hours and then washed and examined. Other leaves were similarly treated in equal concentrations of Chrysoidine Y.

The approximate amounts of free water in the fat cells were determined by placing leaves in various concentrations of calcium chloride and recording the degree of plasmolysis. Concentrations used were 0.05 M, 0.10 M, 0.15 M, — 0.40 M. Both green cells and fat cells were measured using as units those on an ocular micrometer. No attempt was made, nor was it necessary, to convert the units into microns or any other standard unit. The formula used for calculating the amount of plasmolysis was:

$$g = \frac{L - 1/3 D}{H}$$

where L=length of protoplast after plasmolysis

D=diameter of cell

H=length of protoplast before plasmolysis

g =% original volume remaining after plasmolysis (Höfler).

For each concentration, 10 or 20 or sometimes 30 cells of each type (green cell and fat cell) were measured, g was calculated and an average g was found. Then the average g for each concentration was plotted against the logarithm of the concentration as shown in Figure 1.

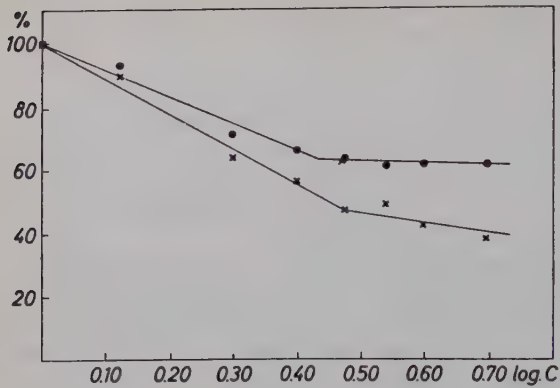


Figure 1. *Plasmolysis curves of green and fat cells of Elodea.* On the abscissa log. concentration of plasmolyticum, on the ordinate cell volume in % of original volume. ● = Fat cells, × = green cells.

The pH of the cell sap was determined as nearly as possible, using Small's Range Indicator Method (Small). Results were difficult to interpret because the indicators did not always penetrate the cells. The list of indicators used is to be found in the Results section in Table 1.

Tests were made for the presence of several common enzymes and their substrates, in order to determine something of the nature of the lipids present. Tests for lipase and acid and alkaline phosphatase were made using the methods of Gomori as described by Lillie. In each case, controls were made by omitting substrate from the incubation media. Tests were also made for substrates to these enzymes by adding small amounts of each of the enzymes to the media and omitting substrates.

3. Results

When fresh, untreated leaves of *Elodea* were examined under bright field, there appeared to be present many randomly distributed cells which at first seemed empty. No nuclei were visible, plastids were not present and streaming was undetectable. However, when the leaves were placed in a hypertonic solution, these "empty" cells were seen to plasmolyze. Under these conditions, the protoplast was highly refractive, suggesting lipids. No further detail was visible. When these fat cells were examined with phae-contrast, streaming of cytoplasm was sometimes observed. The cytoplasmic layer seemed quite thin.

When conditions of drying or injury were such that the cells in the leaves became moribund, the fat cells at first became finely granular around their peripheries. This granular effect then quickly spread throughout the cells. This condition was taken as an indication of death since the cells became rigid and could no longer be plasmolyzed or deplasmolyzed. Consistent variations also took place. Sometimes globules appeared within the protoplast.

Table 1. *Color reactions of green cells and fat cells of Elodea.*

Indicator	Acid color	pH less than	Alkaline color	pH more than	Green cells	Fat cells
Brom thymol blue	yellow	6.2	blue	6.4	yellow	yellow
Brom cresol purple ...	yellow	5.9	purple	6.2	yellow	(red)
Methyl red	red	5.2	yellow	5.6	yellow	red
Brom cresol green	yellow	4.0	green	4.4	(pale green)	yellow
Brom phenol blue	yellow	3.4	blue	4.0	(green cyto)	(yellow)
Methyl orange	red	2.0	orange	4.0	—	orange

These globules were clear and refractive while the remainder of the protoplast was granular. Now and then, upon death, the protoplast separated into two polar "caps" separated by a rather enlarged central section which was globular.

When leaves were treated for protein using the potassium-ferricyanide-ferric chloride method, nuclei in the green cells usually assumed a pink color. They were occasionally pale blue. Fat cells also became pink. Infrequently a blue fat cell appeared. Dead fat cells were granular. There was no clear definition within the fat cells of precisely where the color was deposited. When leaves were treated with the tannin-ferric chloride method, all fat cells appeared black or grey. A few were blue-black and a few were pink. Some cells had caps, some were granular and some were clear. Nuclei in green cells were grey-black.

Aceto-carmin was excellent for demonstrating nuclei in the fat cells as was Buffalo Black. Nuclei were nearly always pressed into corners of the cells and frequently they were irregular and even appeared to be disintegrating. In many fat cells no nuclei could be found.

When fat cells were treated with Schiff reagent, (reduced basic Fuchsin), the entire fat protoplast became brilliantly stained in only a few minutes. When the leaves had first been de-fatted with alcohol or ether, the reaction was almost completely absent. A slightly stronger reaction occurred in those leaves which had been defatted in acetone and then treated with Schiff reagent. Starch was absent from the fat cells, as were tannins.

Sudan III, Sudan IV and Sudan Black B all were taken up very well by these cells. The best dye solvent was ethylene glycol. The dyes were not sufficiently soluble in water and alcohol dissolved out the fatty materials from the cells before they could become stained. The entire protoplast became colored. Nile blue A was taken up very well by clear fat protoplasts but not by granular ones. The contrary was true of the Sudan dyes. Osmium tetroxide was quickly reduced to metallic osmium and gave the same appearance to the fat cells as when they had been stained with Sudan Black B.

Many of the fat cells were readily vitally stained by Neutral Red. The cells varied in color between orange and pink. A considerable number of cells, however, did not take up the stain at all. Chrysoidine Y, at equally dilute concentrations, was never visibly taken up by the fat cells. Concentrations of Chrysoidine in the external medium sufficient to color the cells, were always fatal.

When the leaves were plasmolyzed in increasingly higher concentrations of calcium chloride, it was clear that the fat cells were more resistant to plasmolysis than the green cells. Beyond a certain concentration, no greater plasmolysis of the fat cells could be induced, even though the green cell protoplasts would continue decreasing until practically all water was removed. When the logarithm of the concentration was plotted against the percent volume of the protoplast remaining after plasmolysis, it was found that approximately one-half the cell volume of the fat cell was resistant to plasmolysis.

Upon attempting to determine the pH of the cells, it was generally found that penetration of the cell was most rapid when the pH of the indicators had been adjusted such that they were within their so-called effective range. When a particular indicator did enter the cells, its color was sometimes so pale (usually yellow) that it could not be seen. If this happened to be the indicators acid color, the cells were then flooded with a weak base and the basic color became apparent. Such a technique demonstrates the presence of the indicator where it otherwise could not be observed. The pH of the green cell vacuole was determined to lie somewhere between 5.6 and 5.9. The pH of the fat cells was about 4.0.

Tests for lipase were strongly positive. All fat cells became grey to black. Green cells were not affected. Some fat cells were finely granular, some were globular and some were separated into caps. In this latter instance, the caps were always colored less intensively than the central "vacuolar" area. Tests for lipase substrate were equally strong and indistinguishable from tests for lipase. Controls were likewise strongly positive. Little difference in reactions could be observed between fresh leaves and leaves killed and fixed before testing for lipase.

When testing for acid phosphatase, the results were always clearer on dead leaves. The problem is apparently one of penetration. Some fat cells became thoroughly blackened as a result of the tests for acid phosphatase, while most of them remained unaffected. Green cells were generally gray. When testing for substrate, results were equally lacking in emphasis. Green cells were grey throughout the leaf. Fat cells usually appeared uncolored, but some of them were grey and rarely a black one was found. Controls closely resembled the tests for acid phosphatase substrate.

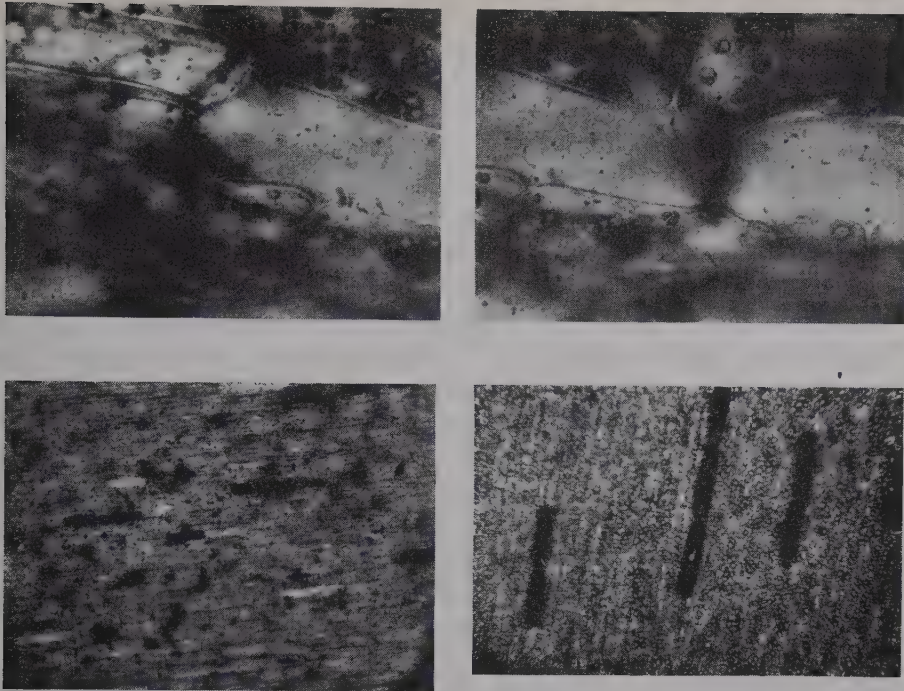


Figure 2. Top: Photomicrograph showing green and non-green cells of *Elodea*. $\times 485$. Bottom left: *Elodea* leaf treated with Sudan Black B. Note that some non-green cells take up heavy concentrations of this dye while others do not stain at all. $\times 215$. Bottom right: *Elodea* leaf treated by Gomori method to demonstrate lipase. Non-green cells show much higher concentrations than green cells. $\times 250$.

Alkaline phosphatase tests usually caused fat protoplasts to become black and green cells to become grey. Again, killed leaves gave clearer results. Sometimes the fat cells were black, sometimes they were grey, but they were consistently darker than the green cells. Substrate tests were less positive but still very similar to the tests for the enzyme. Control leaves were identical to leaves tested for substrate. Figure 2 shows results of tests on *Elodea* leaves for fats (Sudan Black B), lipase (Gomori method), and the presence of nuclei in fat cells (Buffalo Black).

4. Discussion and Conclusions

The present investigation demonstrates the occurrence of idioblastic cells in the leaves of *Elodea*. It would appear that these cells contain large amounts of lipids which are associated with proteins. When the cells die, they may

actually be observed to separate from a clear condition into two separate phases consisting of clear globules and a surrounding granular matrix. It is presumed that the globules are the fat and that the granular matrix is the newly denatured protein. There is good reason to believe that the fat is a phospholipid since the fatty material was relatively insoluble in acetone and because of the positive results when testing for acid and alkaline phosphatase substrates.

Always present is the problem of penetration. Neutral Red, for example, frequently penetrated some fat protoplasts quickly. In fat cells near by, staining might not occur. This resembles the results obtained by many German workers on onion epidermis and Tulip epidermis (Flasch, Höfler and Schindler). However, they were able to show a relationship between accumulation of Neutral Red and the presence of tannins in the cell sap. No such correlation is possible here. The results must then be interpreted in terms of permeability. Precisely why neighboring cells should differ so greatly in permeability is unknown.

The presence of a vacuole, as vacuoles are commonly considered, is highly unlikely. Plasmolysis has shown that much of the volume of the fat cells was non-aqueous. It would appear that these idioblasts possess an extremely thin layer of cytoplasm, which is hardly more than a limiting membrane and that the central area of the protoplast consists of a phospholipid-protein complex. Further evidence in support of this suggestion is the fact that no cytoplasm has ever been observed except with phasecontrast and then the streaming was very difficult to see. And that the nuclei, when observed, were always closely appressed to the cell wall, even flattened. The only alternative to this would be that the entire protoplast is cytoplasm and that no real vacuole exists. Either suggestion is sufficiently bizarre to warrant caution.

5. Summary

It has been shown that in the leaves of *Elodea densa*, in addition to the normal, green cells, there are idioblastic cells which possess the following characteristics:

1. Nuclei are often irregular or flattened and appressed against the sides of the cells.
2. Plastids are totally absent.
3. Only one-half of the cell volume contains removable water, as compared to the green cells of which nearly the whole volume is water.
4. The pH of the "vacuole" is considerably lower than that of the green cells.

5. Lipids are present in large amounts, probably as phospholipid-protein complexes.
6. Enzymes demonstrated as being present include lipase and acid and alkaline phosphatases.

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Recherches comparatives sur la respiration des phases gamétophytique et sporophytique du développement chez *Allomyces*

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Introduction

L'obtention de cultures gamétophytiques et sporophytiques séparées d'*Allomyces* en milieu liquide synthétique (Turian 1955 a, Machlis et Crasemann 1956) a ouvert la possibilité à des recherches comparatives sur leur métabolisme.

Dans cette première étude, nous nous sommes proposés de comparer la respiration de colonies gamétophytiques (G) et sporophytiques (S) d'*Allomyces macrogynus* Emers., d'âge physiologique comparable selon le critère d'une correspondance entre le degré d'acidité (pH) des filtrats de cultures et le degré de développement des colonies mycéliennes. Entre temps, Bonner et Machlis (1957) ont publié les résultats de leurs intéressantes recherches sur la respiration mycélienne et mitochondriale d'*Allomyces macrogynus* mais limitées à la seule phase sporophytique du développement de la Moisissure.

Dans nos premières séries d'essais, les mesures respiratoires ont été effectuées dans le milieu même de culture d'*Allomyces*, le milieu synthétique liquide de Machlis glucosé, afin de maintenir les mycélias dans leurs conditions initiales de culture et de pouvoir ainsi comparer la respiration des hyphes gamétophytiques ou sporophytiques en croissance active (§ 1). Le transport des mycélias en solution saline diluée aurait par contre stoppé la croissance des hyphes et amorcé les processus de la différenciation gamétangiale ou sporangiale des apex hyphaux (Turian 1955 a et 1957); nous

avons mesuré, dans ces conditions, des variations respiratoires caractéristiques (§ 3). D'autres essais ont été consacrés aux effets inhibiteurs éventuels de l'arsénite sur la respiration des jeunes mycélias des deux phases (§ 2), à la comparaison du pouvoir réducteur d'extraits G et S (§ 4) et à l'analyse comparative des acides organiques libérés par les mycélias G et S dans leurs filtrats respectifs (§ 5).

Techniques

Nous avons cultivé les deux phases de développement d'*Allomyces macrogynus* en milieu liquide synthétique B de Machlis (1953), glucosé à 0,5 %, à 25°C (voir Turian 1957). Pour apprécier l'action des substrats (glucose, acétate) sur la respiration des mycélias en solution saline (§ 2), nous avons dû abaisser le taux du milieu en glucose de 0,5 % à 0,05 %, ce qui a permis de réduire quelque peu l'intensité de la respiration endogène, selon les indications de Bonner et Machlis (1957).

L'inoculation des flacons (erlenmeyers Pyrex) contenant 25 ml. de milieu (CaCl_2 et glucose stérilisés séparément) a été réalisée avec 2 types de suspension stérile (0,1 ml./flacon) de cellules mobiles d'*Allomyces macrogynus*, soit: 1) des planozygotes résultant de la fécondation des gamètes libérés à partir d'un petit bloc de jeune culture gamétophytique sur milieu amidon — extrait de levure (Emerson 1941, voir Turian 1957) et immergé 3—4 h. dans 5 ml. de solution DS (composée des sels minéraux principaux du milieu de Machlis dilués 10×); 2) des zoospores «réduites» ou méiospores libérées d'un bloc de sporophyte porteur de sporanges de résistance âgés d'au moins 2 mois (maturation assurée) et plongé pendant 5—6 h. dans 5 ml. de solution DS, à 25°C.

Pour la germination rapide et normale de ces cellules gaméto- ou sporophytiques, nous avons généralement dû omettre l'addition d'acide borique, trop toxique pour les plantules d'*Allomyces*. L'adjonction de cet agent anticopulant (Turian 1955 a) s'est par contre révélée nécessaire pour le maintien et le transfert du mycélium gamétophytique pur en culture stationnaire prolongée.

Les flacons ensemencés ont été placés sur un agitateur (75 secousses par minute) placé dans une étuve à 25°C. Dans ce milieu agité, les plantules gamétophytiques (G) issues des méiospores et les plantules sporophytiques (S) nées des planozygotes se développent sous forme de boules ou sphères mycéliennes, de diamètre assez inégal. Pour les mesures respiratoires comparées de la première série d'expériences (§ 1), nous avons choisi les plus grosses boules, si possible de même diamètre et d'âge physiologique correspondant sur la base des critères suivants: décalage chronologique de 48 heures environ dû à la durée prolongée du lag gamétophytique; acidité des filtrats, mesurée par le pH, proportionnelle à l'âge physiologique des cultures. Le choix des plus grosses boules permet d'éviter la confusion possible, dans le cas du gamétophyte cultivé en l'absence d'acide borique, avec les petites boules sporophytiques d'origine secondaire (libération et copulation gamétiques). D'autre part, si la forme sphérique des mycélias n'est peut-être pas très favorable à une grande intensité des échanges gazeux (Darby et Goddard 1950), elle présente par contre l'avantage, sur le plan comparatif, de permettre un choix de boules mycéliennes gaméto- ou sporophytiques tant morphologiquement que pondéralement comparables (diam. 4—5 mm., poids sec 2—3 mg. pour les cultures G_7 ou S_5 par

exemple, voir plus bas). Ces grosses boules ont été transférées, à l'aide d'une paire de pincettes à mors plats et lisses, en évitant de les serrer, dans du milieu de Machlis glucosé neuf. Après lavage dans ce milieu neutre, elles ont été introduites, à la pincette, dans 3,0 ml. de milieu de Machlis glucosé 0,5 %, préalablement pipeté dans l'espace annulaire des cuves de Warburg contenant 0,2 ml. de KOH 10 % (ou d'H₂O dist.) dans le godet central.

Pour les études comparatives sur l'inhibition de la respiration par l'arsénite (§ 2), nous avons utilisé des plantules plus jeunes: cultivées 48 et 56 h. pour S, 64 et 90 h. pour G, en milieu de Machlis glucosé 0,05 % et enrichi en acide L-glutamique 10⁻⁴ M (pour réduire le lag selon Machlis et Crasemann 1956). Les plantules ont été recueillies sur entonnoir garni d'une toile de nylon. Après 3 lavages successifs avec une solution tampon phosphates neutre (KH₂PO₄—K₂HPO₄ 0,04 M), elles ont été recueillies dans un cristalliseur contenant une solution saline diluée composée de CaCl₂ 0,0005 M—MgCl₂ 0,0005 M—KH₂PO₄ 0,015 M, pH 7,0. Une pipette calibrée à large ouverture (5 mm. diam.) a permis d'introduire 2,8 ml. de suspension saline de plantules dans l'espace annulaire des cuves de Warburg. La solution (0,2 ml.) de substrat (2 mM de glucose en concentration finale), avec ou sans inhibiteur arsénieux, a été placée dans l'ampoule latérale des cuves et la potasse (0,2 ml. KOH 10 %) dans le godet central.

Enfin, pour l'étude des variations respiratoires des mycélias en voie de différenciation gamétangiale (§ 3), nous avons cultivé le gamétophyte d'*Allomyces* pendant 5 j. sur milieu de Machlis glucosé 0,5 % + H₃BO₃ 1/600 M, à partir d'un inoculum mycélien et en culture stationnaire. Dans ces conditions, le mycélium G forme une colonie étendue d'hyphes indifférenciés, disposés parallèlement les uns aux autres sur la périphérie de la colonie. Il est ainsi facile de prélever des fragments de ce mycélium (env. 8 mm. long et 2 mg. poids sec) composés d'un nombre suffisant d'hyphes indifférenciés. Ces fragments, lavés 2× dans la solution tampon (voir plus haut), ont été introduits dans 3,0 ml. de solution saline diluée de Machlis (sol. DS) distribuée dans les cuves de Warburg. Nous les y avons laissés 2 1/2 h. supplémentaires (= 3 h. depuis le prélèvement), à 25°C, après quoi, l'agitation de l'appareil de Warburg a été mise en marche. Deux cuves ont été sacrifiées pour permettre le contrôle microscopique périodique du déroulement des stades de la différenciation gamétangiale.

Toutes les mesures manométriques ont été réalisées avec agitation circulaire (120 oscillations/minute), à 25°C, selon la méthode directe de Warburg, avec les corrections nécessitées par la rétention du CO₂ dans le milieu.

Les échanges gazeux ont été rapportés à l'unité de poids sec (mg.) des boules mycéliennes récupérées à partir de chaque cuve, à la fin des mesures respiratoires. Il est en effet impossible de standardiser la quantité pondérale de matériel mycélien introduit dans les cuves car les boules diffèrent de volume. Ces dernières ont été séchées à 103°C jusqu'à poids constant et pesées à la balance analytique (type semi-micro Mettler). Le poids sec total des boules n'a jamais dépassé 8 mg. par cuve de Warburg.

Des extraits mycéliens G et S ont été préparés par broyage de boules de même diamètre dans un petit mortier d'agate, dans un même volume (5 ml.) de solution tampon phosphates 1/15 M de pH 6,6, en présence de sable de quartz. Le surnageant, après centrifugation à 4000 t./min., a été testé pour son pouvoir réducteur des colorants rédox (thionine surtout), en présence de substrats carbonés, dans des tubes de Thunberg vidés de leur air avec la trompe à eau (§ 4).

Le pH des filtrats a été déterminé avec le titriscope Metrohm et la méthode de la chromatographie sur papier nous a permis de séparer les acides organiques libérés

dans le milieu par les deux phases de développement d'Allomyces (§ 5). Pour cela, une quantité connue de filtrat a été séchée sur papier Schleicher et Schuell No 2043 b. Après développement unidimensionnel descendant au mélange butanol-acide formique- H_2O (butanol saturé d'eau +1 % $HCOOH$) et séchage à $80^{\circ}C$, les taches d'acides ont été révélées avec le bleu de bromophénol. Pour les acides cétoniques, nous avons utilisé le butanol saturé d'eau comme solvant et la révélation des dinitro-phénylhydrazones en lumière UV (Linskens, 1955).

Résultats

§ 1. *Mycélias en croissance active*

Pour une comparaison valable des mesures respiratoires effectuées sur les deux phases de développement d'Allomyces, il était nécessaire de tenir compte du retard initial de la croissance gamétophytique (lag prolongé lors de l'émission de l'hyphe de germination des méiospores) afin de n'utiliser que des cultures de même âge physiologique et non simplement chronologique.

Lors de sa croissance mycélienne S, Allomyces détermine une acidification progressive de son milieu par production d'acide lactique (Ingraham et Emerson 1954). Nous avons montré que les deux phases S et G sont productrices d'acide lactique (§ 5) et cela, dans des proportions sensiblement égales (déterminations titriscopiques inédites, Turian 1957). Il nous est donc apparu que le degré de cette acidité, mesuré par le pH des filtrats, pourrait fournir un critère de l'âge physiologique de nos cultures G et S. Nous avons pu déterminer que l'acidification comparable des filtrats G et S correspond à un décalage d'environ 2 j. dans l'âge chronologique des cultures G par rapport aux cultures S. Ce sont en effet les cultures G âgées de 7 j. et les cultures S de 5 j. qui nous ont donné la meilleure correspondance des pH, soit $G=4,35$ et $S=4,25$. A noter que cette différence de 48 h. dans la vitesse de développement des deux phases correspond sensiblement à la différence des durées respectives des lags G et S (voir Machlis et Crasemann 1956).

Lors de nos mesures respiratoires, ce sont les cultures G de 7 j. (G_7) et S de 5 j. (S_5) qui, en milieu de Machlis glucosé 0,5 %, nous ont montré la plus nette différence de consommation d' O_2 (fig. 1). Les boules mycéliennes utilisées étaient de diamètre comparable et présentaient des poids individuels de 2—3 mg. secs. Les boules des cultures G_6 — S_4 étaient encore trop petites pour être correctement choisies à la pincette (voir techniques). Quant aux couples comparatifs G_8 — S_6 et surtout G_9 — S_7 , leurs échanges gazeux étaient déjà nettement ralentis par l'autolyse naissante dans les hyphes (fig. 2).

La détermination des quotients respiratoires a fourni des valeurs étonnemment basses, soit 0,5 pour G_7 et S_5 . Une importante synthèse de lipides se manifeste visiblement dans les hyphes. On doit aussi tenir compte d'une cer-

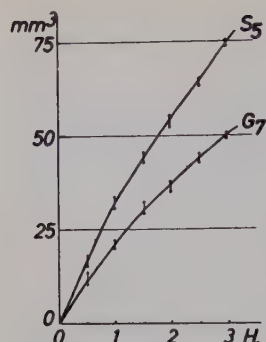


Figure 1. *Respiration comparée des boules mycéliennes gamétophytiques âgées de 7 j. (G₇) et sporophytiques de 5 j. (S₅) d'Allomyces macrogynus en milieu synthétique liquide de Machlis glucosé 0,5 ‰. Ordonnée: consommation d'O₂ mm³.*

taine rétention de CO₂ à l'intérieur des boules mycéliennes (Darby et Goddard 1950, Crasemann 1957).

La pureté gamétophytique des boules G utilisées pour les mesures respiratoires a été vérifiée par l'observation microscopique des organes de reproduction — couples de gamétanges — différenciés en 7—8 h. en solution saline diluée ou simplement eau distillée (Turian 1957) sur les hyphes périphériques de boules de même diamètre prélevées dans le même flacon que celles transférées dans les cuves de Warburg. Nous avons ainsi pu contrôler que toutes les grosses boules G₇ par exemple (3—5 mm. diam.) ont différencié des gamétanges. Les petites colonies sporophytiques provenant d'une éventuelle libération secondaire de gamètes n'ont pas eu le temps de rattraper la croissance des boules gamétophytiques.

Comme expérience-contrôle supplémentaire, nous avons inoculé des méiospores en milieu de Machlis glucosé 0,5 ‰ + H₃BO₃ 1/1200 M et 1/600 M afin de retarder au maximum toute croissance S secondaire. La croissance des plantules G a été elle-même fortement ralentie, surtout en présence de H₃BO₃ 1/600 M. Il a fallu 14 j. de culture sur Machlis boriqué 1/600 M pour obtenir des boules de 1—2 mg. dans un filtrat de pH 5,4. La consommation cumulative d'O₂ de ces boules G₁₄ s'est élevée à la moyenne de 50 mm³ O₂/3 h. Cette

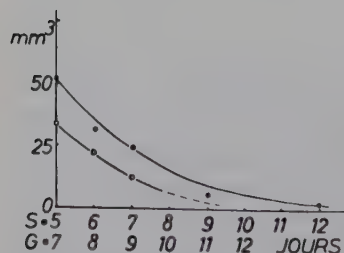
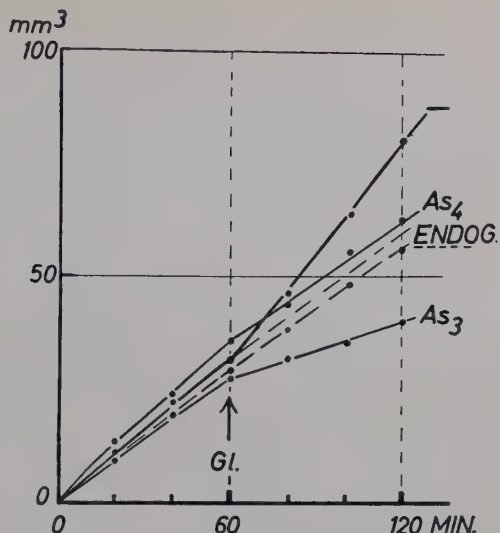


Figure 2. *Effets comparés du vieillissement sur la consommation d'O₂, mesurée après 2 h., des boules mycéliennes gamétophytiques (G) et sporophytiques (S) d'Allomyces macrogynus en milieu de Machlis glucosé 0,5 ‰.*

Figure 3. *Respiration endogène (Endog.) du gamétophyte (âge 64 h.) et effet de l'addition du glucose (2 mM), en l'absence (—) ou en présence d'arsénite de Na $5 \cdot 10^{-3}$ M (As_3) et $5 \cdot 10^{-4}$ M (As_4). Ordonnée: consommation d'O₂ mm³.*



consommation, sensiblement égale à celle des boules G₇ (fig. 1), est restée bien inférieure à celle de boules S comparables ($pH \geq 4,25$).

Il ressort donc de cette première série d'expériences que, sur milieu de Machlis glucosé 0,5 %, propice à la croissance hyphale, le sporophyte d'*Allomyces macrogynus* consomme davantage d'O₂ que le gamétophyte d'âge physiologique correspondant: cette augmentation s'élève à 50 % dans les conditions favorables de comparaison G₇—S₅.

§ 2. Inhibition respiratoire comparative par l'arsénite

En conformité avec les résultats obtenus par Bonner et Machlis (1957) avec le sporophyte d'*Allomyces*, l'arsénite s'est révélé un efficace inhibiteur de la respiration des deux phases G et S du développement chez *Allomyces macrogynus*. Contrairement à notre attente, son pouvoir inhibiteur ne s'est cependant pas montré sensiblement différent à l'égard de la respiration des deux phases: environ 50 % inhibition du taux initial de consommation d'O₂, en présence de $5 \cdot 10^{-3}$ M de AsO₂Na (fig. 3 pour phase G).

Le glucose n'a que faiblement stimulé la consommation d'O₂ après son introduction dans la suspension de plantules en solution saline (fig. 3 et techniques). Cette stimulation, représentant la respiration exogène, est du même ordre de grandeur (env. 20—25 % du taux initial de respiration endogène) pour les phases G et S.

Avant de sécher les mycélias en vue de la détermination de leur poids sec, nous avons effectué un rapide contrôle microscopique de leur état de

différenciation. Les mycélias G ayant séjourné env. 3 h. dans le milieu faiblement glucosé (2 mM) + arsénite $5 \cdot 10^{-4}$ M et même $5 \cdot 10^{-3}$ M étaient porteurs d'un grand nombre de gamétanges en voie de différenciation, avec une forte proportion de gamétanges mâles terminaux isolés sur des gamétanges femelles indifférenciés et vacuolés (effet «androgène» de l'arsénite, Turian 1957). Les mycélias S, porteurs de jeunes zoosporanges sur les témoins faiblement glucosés, exhibaient par contre, en présence supplémentaire d'arsénite, des sporanges de résistance plus ou moins typiques, dépourvus de papilles mais avec une membrane épaissie et brunâtre. Ces variations imprévues de la morphogénèse d'*Allomyces* ne sont probablement pas étrangères à la similarité de la réponse respiratoire de ses deux phases de développement à l'action toxique de l'arsénite (voir discussion).

§ 3. *Corrélations entre respiration et différenciation sexuelle gamétangiale*

Nous avons mis à profit la rapide différenciation gamétangiale sur les mycélias G placés en solution saline diluée (voir techniques) pour suivre les variations de l'activité respiratoire en fonction du déroulement des stades successifs de cette morphogénèse sexuelle chez *Allomyces* (pour la description complète de ces stades, voir Turian 1957).

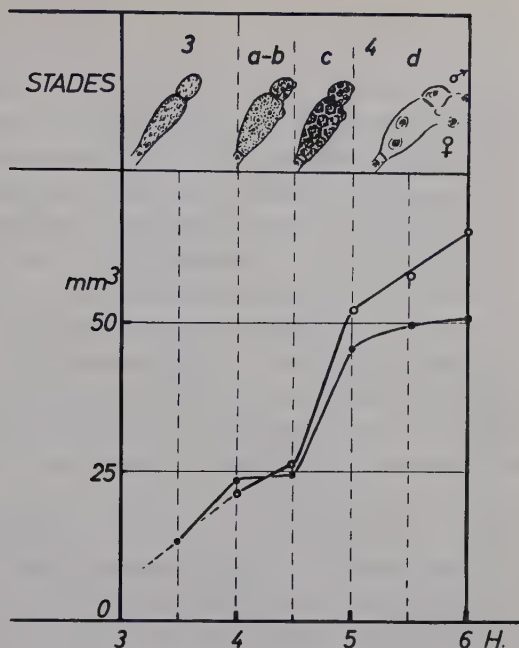
Il ressort de l'examen des courbes respiratoires obtenues avec ces fragments mycéliens G (fig. 4) qu'une nette accélération de la respiration est intervenue dès le début du stade de clivage intra-gamétangial (stade 4_c, Turian 1957). Cette élévation de l'intensité respiratoire, faisant suite à une période stationnaire, correspondrait ainsi avec l'apparition des masses basophiles organisatrices de corps paranucléaires ribonucléoprotéiques distincts dans les unités gamétiques en voie d'individualisation dans les gamétanges.

La comparaison des activités respiratoires des mycélias G en voie de différenciation n'a donné que de faibles différences selon que les mesures ont été effectuées en présence de KOH ou d'H₂O dans le godet central des cuves de Warburg. C'est là l'indication d'un Q.R. CO₂/O₂ peu élevé, de valeur imprécise (env. 0,25) par suite de différences d'amplitude des courbes données par les différents fragments mycéliens étudiés.

§ 4. *Pouvoir réducteur des extraits mycéliens*

La méthode des tubes de Thunberg par la réduction de colorants rédox en présence d'homogénats et de substrats respiratoires a permis d'obtenir un premier résultat différentiel: les extraits de la phase S ont réduit plus rapidement le bleu de méthylène et surtout la thionine, en présence de succinate comme substrat, que les extraits G. Dans nos essais préliminaires, le citrate n'a pas fonctionné comme donneur d'H₂, aussi bien avec les extraits G que S, en présence de bleu de méthylène.

Figure 4. Correspondance entre les variations respiratoires et les derniers stades de la différenciation gamétangiale (Turian 1957) du gamétophyte d'*Allomyces macrogynus* maintenu en solution saline diluée. Ordonnée: consommation d'O₂ mm³.



§ 5. Acides organiques des filtrats

Par la méthode de la chromatographie sur papier (voir techniques), nous avons pu séparer les acides organiques des filtrats G et S. Nous y avons détecté 2 acides principaux: l'acide lactique, au rF 0,8 dans nos conditions, et une tache de rF env. 0,20—0,25 correspondant à un acide «tartro-oxalique». Ce dernier présente en effet des caractéristiques mixtes: coloration rougeâtre avec le test au résorcinol — ac. sulfurique conc. et absence de cristaux en présence de KCl saturé ou d'acétate de K après chauffage à reflux (voir Wattiez et Sternon 1942); tache jaune à rF de l'acide tartrique (comparaison avec acide pur) mais avec caractère «trainant» de l'acide oxalique.

L'intensité de la coloration jaune de la tache «oxalo-tartrique» est comparable à celle de l'acide lactique pour les filtrats G ou S d'*Allomyces* cultivé sur milieu de Machlis glucosé. Son intensité s'accroît avec l'âge des cultures et par conséquent avec l'abaissement du pH des filtrats (cultures S₉, pH 3,35). L'étendue et l'intensité des taches d'acides nous est apparue comme égale sur les chromatogrammes réalisés avec des filtrats G et S d'âge physiologique comparable (G₉—S₇ par ex.).

Dans les filtrats G et S, nous avons aussi pu déceler la présence de l'acide pyruvique, par la tache principale jaune verdâtre, de rF 0,7, de sa 2,4-dinitro-phénylhydrazone examinée en lumière UV (comparaison avec échantillon témoin d'acide pur).

Discussion

Nous avons montré qu'en milieu physiologique, favorable à la croissance mycélienne, la phase gamétophytique du développement chez *Allomyces macrogynus* se distingue de la phase sporophytique par la réduction de $1/3$ de sa respiration rapportée à l'unité de poids mycélien sec.

On peut par contre calculer que, sur la base de l'unité génômique ($N \rightarrow 2N$ chromosomes) et non plus pondérale (mg. poids sec), l'efficacité respiratoire du gamétophyte est supérieure à celle du sporophyte. En effet, au doublement du nombre chromosomique ($14 \rightarrow 28$ selon Emerson et Wilson 1954) ne correspond qu'une augmentation de $1/2$ de la respiration, ce qu'on peut encore exprimer: $S/G = 2N/N = QO_2 \text{ } 100/QO_2 \text{ } 66$. La consommation d' O_2 du sporophyte n'atteint donc que le 75 % de celle d'un gamétophyte diploïde théorique dont le QO_2 serait calculé par doublement des valeurs du gamétophyte réel.

Des valeurs d'efficacité respiratoire et biosynthétique ont déjà été calculées dans des séries polyploïdes d'autres microorganismes. Il ne s'agissait toutefois là que de lignées ne différant que par un simple doublement de leurs lots chromosomiques alors que chez *Allomyces* le doublement $N \rightarrow 2N$ correspond à l'alternance des générations (modification phénotypique $G \rightarrow S$).

Ainsi, sur la base du taux de synthèse protoplasmique par génôme, Wetherel et Krauss (1956) ont observé que les souches diploïdes de *Chlamydomonas* n'ont qu'une efficacité de 70 % par rapport aux mêmes souches haploïdes. Chez les Levures, au doublement des lots chromosomiques $2N \rightarrow 4N$ ne correspond qu'un accroissement de poids sec de $1,76 \times$ (Duraiswami 1951). Par contre, selon Ogur et coll. (1952), l'accroissement de la teneur cellulaire en ADN, en ARN, en métaphosphates et en poids sec irait, dans les limites de certaines conditions expérimentales, de pair avec l'augmentation du degré de ploidie chez *Saccharomyces*. Il en serait de même pour le QO_2 et le QCO_2 chez cette même levure (Ogur 1954). Rappelons aussi que les levures diploïdes ont été considérées comme étant du type respiratoire alors que les levures haploïdes se rattacheraient au type fermentaire (Gonzalez et Barron 1956).

Nous avons pensé que l'arsénite aurait manifesté une plus grande toxicité à l'égard du sporophyte dans sa phase zoosporangiale (en milieu liquide) qu'à l'égard du gamétophyte (§ 2). Ce dernier, mis au contact de doses élevées d'arsénite, accuse au cours de sa différenciation, une prépondérance de gamétanges mâles (Turian 1957), apparemment moins sensibles à cet inhibiteur que les gamétanges femelles et leurs homologues sporophytiques, les zoosporanges. Or, il s'est trouvé que l'intoxication à l'arsénite oriente le sporophyte vers le sporange de résistance, au détriment de sa seconde possibilité organogénique, le zoosporange. L'effet de l'inhibiteur souligne ici l'homologie

gamétange mâle — sporange de résistance précédemment établie sur une base morphologique (Sörgel 1937). Cantino (1951, 1956) a démontré que chez *Blastocladiella*, genre voisin d'*Allomyces*, la formation du sporange de résistance est liée à une déficience du cycle de Krebs. Il y a donc, dans chacune des phases, une alternative morphogénétique dont chaque composante paraît liée à un métabolisme particulier. L'une d'entre elles échappe largement à l'inhibition par l'arsénite. Nos essais ont montré parallèlement que la respiration du G masculinisé et du S porteur de sporanges de résistance est affectée dans la même proportion par une dose donnée d'arsénite.

En solution saline diluée, la croissance hyphale cesse rapidement au profit des processus de la différenciation reproductive (§ 3). Les variations d'intensité de la respiration endogène correspondent dès lors aux activités métaboliques déterminant la différenciation progressive des gamétanges (G) ou des zoosporanges (S). C'est ainsi que l'élévation tardive du QQ_2 endogène, mise en rapport avec l'apparition des masses basophiles paranucléaires lors du stade de clivage des énergides-gamètes (fig. 4), est vraisemblablement en corrélation avec l'augmentation momentanée de la demande d'énergie nécessitée par la synthèse accrue d'acide ribonucléique dans les gamétanges. On sait, en effet, que l'ARN est l'un des constituants essentiels du corps paranucléaire des gamètes d'*Allomyces* (Turian 1955_b). En outre, il est intéressant de remarquer qu'après une augmentation régulière du taux de QO_2 , correspondant aux synthèses requises par la formation et l'accroissement des jeunes gamétanges, la consommation d' O_2 reste pratiquement stationnaire pendant une période correspondant au stade des couronnes lipidiques (4b). Or, nous avons précisément décrit ce stade comme une étape d'attente et d'organisation interne des gamétanges (prolongée plusieurs jours sur milieu solide et sec), précédant la reprise des activités de synthèse associées au stade de clivage (Turian 1957).

Summary

Certain aspects of the comparative respiratory metabolism of the gametophytic and sporophytic phases of the development in *Allomyces macrogynus* were studied.

Gametophytic (G) or sporophytic (S) mycelial spheres grown in agitated cultures were chosen for similar size and dry weight and assumed to be of comparable physiological age on the basis of similarity in their pH-filtrates. Such a criterion was obtained with G grown 7 days and S grown 5 days, i.e. with a 48 hours chronological age difference corresponding to the prolonged lag of the gametophytic inoculum (meiospores).

In such standardized conditions, in the liquid synthetic medium of Machlis

with glucose 0,5 %, the respiratory activity (O_2 -consumption) of the sporophyte was found to be 1/2 higher, on a dry weight basis, than that of the gametophyte. However, on a genome or chromosomal basis, this represents a 25 % lower respiratory efficiency for the diploid S phase compared to the haploid G phase.

Low exogenous respiration has been produced in the two phases after the addition to a saline solution of a carbon substrate such as glucose. In such conditions, rather high concentrations of arsenite ($5 \cdot 10^{-3} M$) have determined a comparable degree of respiratory inhibition (50 %) in the G and S phases of *Allomyces*. This may be explained by a kind of adaptative defense mechanism based on the possibility for each phase of the development in *Allomyces* of engaging in alternative morphogenetic and arsenite resistant respiratory pathways leading to single male gametangial morphogenesis on G and resistant sporangial, instead of zoosporangial, morphogenesis on S, as microscopical controls of the reproductive organs differentiated on arsenite treated mycelia have confirmed.

The quickly induced reproductive differentiation of the hyphal tips of *Allomyces* when dipped in dilute saline solution has permitted to observe a net correlation between the accelerated O_2 -consumption of the 4—5 hours dipped sexual G mycelia and the increased basophily (future RNA nuclear caps) in the gametangia during the gamete cleavage stage of the sexual morphogenesis. A preceding stationary QO_2 period seems to be correlated with the resting gamete origin stage (crowns of lipid granules).

Extracts of S mycelial spheres have shown higher reducing properties in the presence of thionin and succinate than G extracts. In the same conditions (anaerobic Thunberg method), citrate was a non reducible substrate by S or G extracts.

Comparable amounts of lactic acid, "oxalo-tartric" acid (low rF) and pyruvic acid were found by chromatography in the G and S filtrates.

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Effect of Various Nitrogen Sources on Growth of Isolated Roots of *Pinus serotina*

By

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Introduction

Studies of the growth of isolated pine roots have been reported by Slankis (1947, 1948 a, 1948 b, 1949 a, 1949 b, 1950, and 1951), working with *Pinus silvestris*, and by Isikawa (1956), working with *Pinus densiflora* and *Pinus Thunbergii*. Neither investigator has reported on extensive investigations of nutritional requirements. Slankis (1948 b) has shown with isolated *Pinus silvestris* roots that exudates of certain mycorrhizal fungi stimulate the formation of numerous dichotomously-branched laterals. In subsequent experiments he has shown (Slankis 1949 b, 1950, and 1951) that indoleacetic acid and naphthaleneacetic acid stimulate dichotomous branchings similar to those caused by fungal exudates, and has suggested a possible role of auxins in mycorrhizal-root relationships.

In a study of isolated roots of pond pine (*Pinus serotina* Michx.) it was demonstrated (Barnes and Naylor 1958) that neither IAA nor NAA, at the concentrations found to be most effective by Slankis, stimulated dichotomous branchings. Several other compounds, notably certain B-vitamins and amino acids, did, however, stimulate the formation of dichotomously-branched laterals when the compounds were added to the standard medium used for growing pond pine roots. Preliminary trials indicated that some of the amino acids involved in the Krebs-Henseleit (1932) urea-ornithine cycle

might be effective in causing dichotomous branchings. The present paper reports on studies of the effects of several nitrogen compounds when used as the sole nitrogen sources for growing isolated pond pine roots.

Materials and Methods

Clonal roots of pond pine were used in all tests. The roots were maintained on White's (1943) nutrient medium, modified (Barnes and Naylor, 1958) as follows: glycine omitted; concentration of $\text{Fe}_2(\text{SO}_4)_3$ reduced to one-half (1.25 mg./l.); thiamine reduced to one-half (0.05 mg./l.); and sucrose concentration increased to 7 per cent. In addition, in order to test the effects of various nitrogen sources, the $\text{Ca}(\text{NO}_3)_2$ in Whites' medium was replaced by CaCl_2 , and the KNO_3 was omitted. γ -aminobutyric acid, arginine, aspartic acid, citrulline, glutamic acid, ornithine, and proline were tested individually for their ability to act as sole nitrogen sources. Those amino acids which appeared to have some capacity to act as a substitute for nitrate were then tested in concentration series. The control medium for tests of nitrogen sources was that as described above, with NO_3^- added (as the Ca^{++} salt) at the concentration used by White, $2.4 \times 10^{-3} M$.

Root segments were grown in 50 ml. of nutrient media in 125 ml. Erlenmeyer flasks. The media were adjusted to pH 5.4 and sterilized by autoclaving. Growth measurements were made after 30 days.

Results

The results of a test of seven amino acids as sole nitrogen sources, when used at a concentration of $10^{-3} M$, are given in Table 1. Pond pine roots having citrulline and γ -aminobutyric acid as sole sources of nitrogen grew almost

Table 1. *Effect of various amino acids (as sole nitrogen sources) on growth of isolated roots of pond pine. Five roots per treatment. Length of test 30 days.*

Nitrogen source	Increase in number of all laterals	Increase in number of living laterals	Growth in length of all tips (mm.)	Branching habit
Nitrate ($2.4 \times 10^{-3} M$) ...	12.0	11.2	158	Normal
None	11.7	7.7	37	Laterals shortened
Amino acids (all at $10^{-3} M$)				
γ -aminobutyric acid ...	16.0	10.3	103	Dichotomous
Arginine	8.3	7.0	35	Tips swollen
Aspartic acid	10.2	9.8	32	Tips swollen
Citrulline	14.2	11.2	119	Dichotomous
Glutamic acid	4.2	— 1.2 ¹	25	Normal
Ornithine	5.7	3.2	17	Tips swollen
Proline	10.0	3.2	28	Normal

¹ More laterals died than were formed: some of the laterals present on the original culture died during the experiment.

Table 2. *Effect of concentrations of amino acids (as sole nitrogen sources) on growth of isolated roots of pond pine. Five roots per treatment. Length of test 30 days.*

Nitrogen source	Concentration <i>M</i>	Increase in number of all laterals	Increase in number of living laterals	Growth in length of all tips (mm.)	Dichotomous branchings per root
Citrulline	10 ⁻³	41.2	38.8	168	17.0
	10 ⁻⁴	24.3	21.7	138	8.7
	10 ⁻⁵	14.3	9.0	133	0.3
	10 ⁻⁶	10.0	2.0	65	0.3
	10 ⁻⁷	8.8	4.2	84	0.8
	0	9.5	1.2	69	0.8
Nitrate	2.4 × 10 ⁻³	12.6	11.4	228	0.0
γ-aminobutyric acid	10 ⁻²	6.2	5.4	76	2.2
	10 ⁻³	35.5	33.2	190	16.0
	10 ⁻⁴	13.4	12.6	190	4.3
	10 ⁻⁵	10.2	7.2	107	0.8
	10 ⁻⁶	6.2	0.2	128	0.2
	10 ⁻⁷	11.0	6.2	106	0.4
	0	7.6	3.4	109	0.8
Nitrate	2.4 × 10 ⁻³	7.4	6.0	210	0.0
Ornithine	10 ⁻³	19.0	10.0	40	0.0
	10 ⁻⁴	30.5	29.5	95	5.0
	10 ⁻⁵	23.2	17.6	104	1.2
	10 ⁻⁶	21.7	20.3	137	0.0
	10 ⁻⁷	10.0	1.2	73	0.0
	0	9.5	1.2	69	0.8
Nitrate	2.4 × 10 ⁻³	12.6	11.4	228	0.0
Arginine	10 ⁻³	8.0	7.2	44	0.2
	10 ⁻⁴	8.8	7.5	48	0.0
	10 ⁻⁵	20.7	19.0	77	0.0
	10 ⁻⁶	14.4	11.0	108	0.2
	10 ⁻⁷	7.8	5.0	55	0.0
	0	12.5	7.5	64	0.0
Nitrate	2.4 × 10 ⁻³	15.0	14.0	220	0.0
Aspartic acid	10 ⁻³	9.8	9.2	40	0.8
	10 ⁻⁴	10.3	9.4	62	0.2
	10 ⁻⁵	8.7	5.7	56	0.0
	10 ⁻⁶	9.3	7.0	55	0.0
	10 ⁻⁷	6.0	2.5	31	0.0
	0	12.5	7.5	64	0.0
Nitrate	2.4 × 10 ⁻³	15.0	14.0	220	0.0

as much as those receiving nitrate, presence of both amino acids being associated with formation of numerous dichotomous branchings. Arginine, ornithine, and aspartic acid caused the formation of swollen tips on the lateral roots. Glutamic acid and proline did not cause any conspicuous changes in branching habit.

The effects of citrulline, γ-aminobutyric acid, arginine, aspartic acid and ornithine were next studied in concentration series. Results of these tests are presented in Table 2. Citrulline and γ-aminobutyric acid proved to be

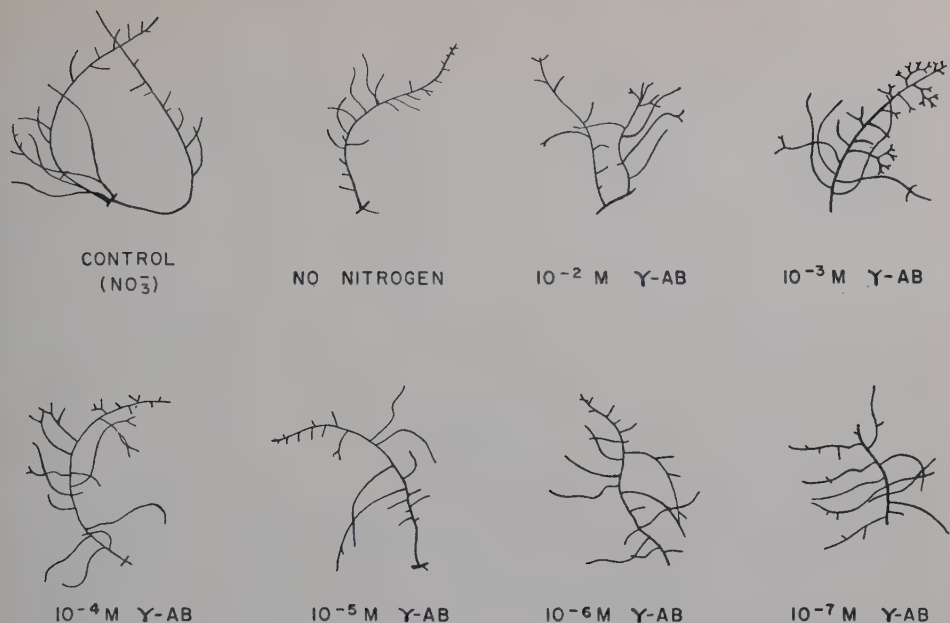


Figure 1. Branching habits of pond pine roots grown with various concentrations of γ -aminobutyric acid (γ -AB) as sole nitrogen sources.

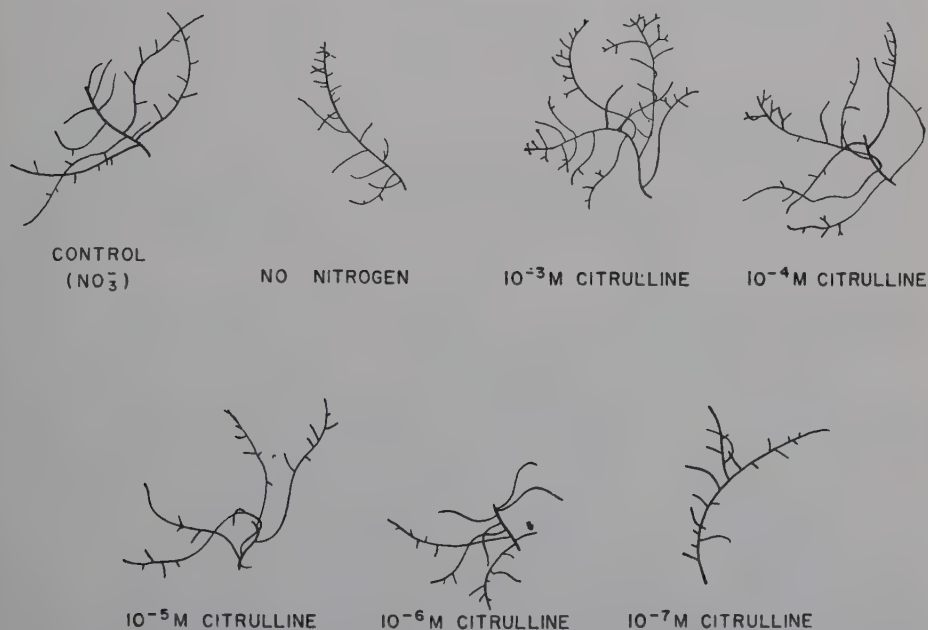


Figure 2. Branching habits of pond pine roots grown with various concentrations of citrulline as sole nitrogen sources.



Figure 3. *Isolated pond pine root maintained through six sub-cultures in medium with 10^{-3} M citrulline as sole nitrogen source.*

excellent sources of nitrogen, while ornithine and arginine were only moderately good sources, and aspartic acid was poor. The most effective concentration of citrulline, both in supporting growth in length and stimulating dichotomous branching, was 10^{-3} M. At the same concentration, γ -aminobutyric acid had similar morphogenetic effects but growth in length was greater and closely approached the nitrate control. Maximum growth in length with ornithine was obtained at 10^{-6} M, while the largest number of dichotomous branchings were noted at a concentration of 10^{-4} M. The concentration of arginine promoting the greatest growth in length was 10^{-6} M. Aspartic acid appeared to be used most effectively at a concentration of 10^{-4} M, although no concentration was more than one-third as effective as the most favorable concentration of γ -aminobutyric acid. Neither arginine nor aspartic acid was effective in promoting dichotomous branchings, although numerous swollen tips were formed in media containing these amino acids.

Illustrations of the effects of citrulline and γ -aminobutyric acid on the growth of isolated pond pine roots are given in Figures 1 and 2. Satisfactory growth rates were maintained by subcultured roots (30-day culture periods for 6 months) grown with 10^{-3} M citrulline or γ -aminobutyric acid as the only nitrogen source. The growth habit of these roots became so completely

Table 3. *Effect of concentration of urea (as sole nitrogen source) on growth of isolated roots of pond pine. Ten roots per treatment. Length of test 30 days.*

Urea (M)	Increase in number of all laterals	Increase in number of living laterals	Growth in length of all tips (mm.)	Dichotomous branchings per root
5×10^{-5}	24.4	19.2	176	3.4
1.5×10^{-4}	18.6	13.9	75	1.6
5×10^{-4}	2.9	0.6	36	0.0
1.5×10^{-3}	3.3	— 11.0 ¹	10	0.0
0	10.5	4.6	53	0.0
Control (2.4×10^{-3} M nitrate)	20.8	19.0	185	0.0

¹ More laterals died than were formed.

changed, however, owing to repeated dichotomous branchings and apparently complete loss of apical dominance, that comparisons with NO_3^- -grown roots could not be made validly. Figure 3 shows a culture maintained through six subcultures on a citrulline medium. Note the striking mycorrhizal mimicry.

Urea was also tested as a nitrogen source for pond pine roots. As shown in Table 3, a concentration of 5×10^{-5} M supported good growth through the first 30-day test. A few dichotomous branchings were also observed. It was found subsequently, however, that the roots could not be maintained on a urea medium — all cultures being dead within 4 months.

Discussion

In general, amino acids have been found to be relatively ineffective as nitrogen sources for growing isolated roots (Street 1957). Previous work with pine species has not been reported. Tryptophane, however, has been shown to be an essential additive in growing rye roots in continuous culture (Roberts and Street 1955). The yeast extract requirement of a Czechoslovakian strain of groundsel (*Senecio vulgaris*) can be substituted for by 2-naphthoxyacetic acid and arginine (Charles 1956). While working with excised roots of another strain of groundsel, Skinner and Street (1954) found that arginine (5 mg./l.) or lysine (2 mg./l.) stimulated root growth when added to the nitrate containing medium. They showed further that these amino acids could function effectively as sole sources of nitrogen for their strain of groundsel. In studies with decotylized pea seedlings (Fries 1954), it was found that glycine, arginine, and glutamic acid supported root growth; and also that arginine could be exchanged for ornithine or citrulline. In the present study with pond pine roots, citrulline was found to be much more effective as a nitrogen source than the other amino acids of the urea cycle. γ -aminobutyric acid, presumably related to the urea cycle only through ornithine and glutamic acid, supported as much or more growth than any other organic nitrogen source. A sufficient number of amino acids was not tested, however, to determine whether close metabolic relationship to the ornithine cycle amino acids is related to the effectiveness of a given amino acid as a nitrogen source for pond pine roots. Urea itself, a product of the ornithine cycle, did not support continuous growth.

The tendency of amino acids to induce dichotomous branching of isolated roots has not been previously reported. When Slankis found (1949 b, 1950 and 1951) stimulation of dichotomous branching of Scotch pine resulted from adding certain auxins to the medium, he did not refer to any tests of the effects of non-auxin compounds. There appears be no obvious relation-

ship between auxin and amino acid metabolism. However, the results observed in other work with pond pine (Barnes and Naylor 1958) indicated that numerous compounds including sulfanilamide, kinetin and pyridoxine caused dichotomous branching. Since these compounds are by no means closely related chemically or metabolically, definite conclusions about the causes of dichotomous branching are not yet possible. The indications are that the physiology of dichotomous branching is very complex, and that approaches other than auxin-dependent ones should be investigated.

Summary

1. Isolated roots of pond pine (*Pinus serotina* Michx.) were grown in sterile culture with γ -aminobutyric acid, arginine, aspartic acid, citrulline, glutamic acid, ornithine, proline and urea as sole nitrogen sources.

2. The most effective organic nitrogen sources supporting growth were found to be citrulline and γ -aminobutyric acid, both at concentrations of 10^{-3} M. Growth in length with these amino acids in the medium was almost as rapid as when nitrate was used.

3. Citrulline and γ -aminobutyric acid (10^{-3} M) also had morphogenetic effects in that they stimulated formation of short dichotomous branches strongly resembling mycorrhizae.

4. Arginine, ornithine, urea and aspartic acid were found to be moderately good to poor sole sources of nitrogen. Although they did cause some change in branching habit of the roots, they did not produce as strong morphogenetic effects as γ -aminobutyric acid and citrulline.

5. Glutamic acid and proline were not effective as nitrogen sources or in promoting dichotomous branching of pond pine roots.

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Demonstration of Cytochrome Oxidase in Onion Root Tips

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Recent reviews by Smith and Chance (25), by James (10), and by Hartree (6), as well as James' earlier book (9), and other reviews by James (8), Goddard and Meeuse (5), and Hill and Hartree (7) illustrate a tendency toward the acceptance of cytochrome oxidase as the general terminal link between the various dehydrogenase systems and oxygen in plants.

Distribution surveys of the various cytochromes and cytochrome oxidase have been conducted by Bhagvat and Hill (3) and more recently by Webster (28, 30). Webster (28) was able to extract an enzyme which could oxidize cytochrome c from the tissues of 54 different species, representing 23 families of dicotyledonous plants. He found this cytochrome oxidase activity to be strongly inhibited by carbon monoxide and the inhibition was reversed by light. In addition to these surveys, many reports establishing the presence of cytochrome oxidase in various plant tissues have appeared (for bibliography see 6, 10, 28). In most cases the cytochrome oxidase was found to be associated with a particulate (mitochondria) fraction of the cell material. All of these results furnish evidence for the general importance of cytochrome oxidase in plant respiration.

This laboratory, as well as several others, has studied certain aspects of the respiration of onion roots (1, 12, 22, 19). It is of interest that Keilin (13) in 1925 reported the cytochrome spectra in eschallot bulb scales as well as in other plant tissues. Bhagvat and Hill (3) reported the presence of cytochromes and cytochrome oxidase in the onion bulb. Martin and Morton (17)

have observed absorption bands of reduced cytochrome (fused spectra of c and b) in whole tissue, and in mitochondria, of apical stems of onions. They have also found cytochrome b_3 in microsomes isolated from onion bulbs (16). Recently, Lott (15) has linked water absorption by the onion root with cellular metabolism, employing carbon monoxide as an inhibitor. Pratley (21), also using carbon monoxide as well as the "Nadi" reagent, has demonstrated the participation of cytochrome oxidase in the origin of bioelectric potentials exhibited by the onion root. In both cases (15, 21) the depressing effects of carbon monoxide were reversible by light. James (11) has spectrophotometrically identified cytochrome oxidase in onion roots. The primary purpose of this report is to demonstrate, by means of respiratory measurements, the presence of cytochrome oxidase in the onion root tip, which apparently has not been recorded.

Materials and Methods

Onion sets were sprouted in aerated quarter strength Hoagland's solution at $25 \pm 1^\circ\text{C}$, and the experimental samples were collected in the manner previously described in detail (2, 18, 19, 20).

Oxygen consumption was measured at $30 \pm 1^\circ\text{C}$ by the Warburg manometric technique using 0.2 ml. of 15 % KOH in the center well as a CO_2 absorbent. With mixtures of gas composed of carbon monoxide and oxygen there is some absorption in KOH in the absence of tissue; therefore a blank was included with each series of determinations and the appropriate correction applied.

All gas mixtures were prepared from commercial gases either by the volumetric-displacement method or the evacuation technique described by Umbreit, Burris, and Stauffer (26). The desired gas atmosphere within the experimental vessels was obtained by the evacuation and replacement technique (26). Control experiments have shown that the actual process of evacuation does not bring about observable differences in gas exchange (18).

Two twenty-watt fluorescent lights were fitted for underwater use and mounted in the Warburg constant-temperature bath allowing a distance of less than one inch between the lights and the bottoms of the reaction vessels. These lights provided an illumination of approximately fifty foot candles at the vessels. When it was necessary for some of the vessels to be in darkness they were wrapped in heavy aluminum foil.

In the experiments employing homogenates the tissue was weighted out and placed in a glass homogenizer tube (26) together with a known amount of buffer. The tissue was then homogenized by the use of a power driven pestle, more buffer was added until the desired volume was obtained, and the homogenate was transferred to the reaction vessels. A known amount of homogenate was denatured (by boiling for five minutes) and placed in a reaction vessel to serve as a control.

Cytochrome c was reduced with sodium ascorbate (24) which was prepared by the addition of 0.1 ml. of 0.1 N sodium hydroxide to 20 mg. of ascorbic acid just before using (26). An additional correction was necessary for the auto-oxidation of ascorbate.

The cytochrome c preparation that was added to the homogenates in some cases was obtained from General Biochemicals and contained 10 mg./ml. In all experiments using cytochrome c, 0.3 ml. was added. Also in these experiments 0.3 ml. of 0.004 AlCl_3 was added and a phosphate buffer at a pH of 7.4 was employed, since these conditions have previously been shown to be optimum for cytochrome oxidase activity (23, 26). In other experiments the phosphate buffer had a pH of 4.5, which undoubtedly limited the activity of the cytochrome oxidase.

Results

Carbon monoxide inhibition of oxygen consumption by apical segments of the onion root: The results of preliminary experiments to determine whether or not carbon monoxide would inhibit the oxygen consumption of apical segments of the onion root tip are recorded in Table 1. The first column shows the oxygen consumed from an atmosphere of 95 % CO and 5 % O_2 , while the second column records the oxygen consumed by an identical sample from an atmosphere of 95 % N and 5 % O_2 , thus serving as a control. It should be noted that 5 % O_2 is not nearly adequate to maintain the maximal rate of endogenous respiration in the onion root tip. Berry and Norris (2) have previously observed that at this temperature (30°C) and under these conditions, a partial pressure of 45 % O_2 is required to support maximal respiration of the 0—5 mm. segments.

It is evident from the last column of Table 1 that CO causes significant inhibition of oxygen consumption.

Carbon monoxide inhibition of oxygen consumption by onion root tips and its reversal by light: It is apparent from the data contained in Table 2 that the rate of oxygen consumption by tissue in the presence of 95 % CO and 5 % O_2 in the dark is significantly inhibited (average 37 %). Identical samples of tissue in the same gas mixture in the light show less inhibition (average 4 %), and in some cases more oxygen uptake occurred than in the

Table 1. *Carbon monoxide inhibition of oxygen consumption by apical segments (0—5 mm.) of the onion root. (Cu. mm. O_2 consumed/100 mg. (wet wt.) tissue/hr.)*

Atm.: 95 % CO 5 % O_2	Atm.: 95 % N 5 % O_2	Per cent inhibition
19	26.8	29
14.2	36.7	61.4
25.7	47.5	46
22	47	53

Table 2. Carbon monoxide inhibition of oxygen consumption by onion root tips (0—5 mm.) and its reversal by light.

Control Atm.: 95 % N ₂ ; 5 % O ₂ mm. ³ O ₂ consumed/100 mg. (wet wt.) tissue/hr.	Experimental Atm.: 95 % CO; 5 % O ₂ mm. ³ O ₂ consumed/100 mg. (wet wt.) tissue/hr.		Percent inhibition or stimulation	
	Dark	Light	Dark	Light
41	30	42	— 27	+ 2
29	26	30	— 10	+ 3
57	38	46	— 33	— 19
63	33	52	— 48	— 17
38	20	31	— 47	— 18
29	11	30	— 62	+ 3
42	29	42	— 31	0
53	34	59	— 36	+ 11
Average 44	28	42	— 37	— 4

control. The stimulation of oxygen consumption shown in some cases in the light is perhaps not too unusual as Webster and Frenkel (29) and Daly (4) have noted stimulation of oxygen uptake by the presence of carbon monoxide. Webster and Frenkel (29) point out that this is apparent at lower CO/O₂ ratios, however Daly (4) in studying the wild plum was using about the same CO/O₂ ratio as is employed in these experiments. By the use of isotopic CO Daly has shown that the increase in oxygen uptake actually results from higher respiratory rates rather than from the oxidation of CO to CO₂.

Effect of carbon monoxide on respiration of onion root tips during alternate dark and light periods: In contrast to the results shown in Table 2, in which one flask was exposed to the light while another was kept in the dark (even though the two flasks contained identical (?) tissue samples), this series of experiments was designed to determine the effects of alternate dark and light periods on the same tissue.

The results of a typical experiment of this type are plotted in Figure 1. It is clear from the slopes of the curves obtained in CO and O₂ that the rate of oxygen consumption is less in the dark than in the light, thus demonstrating the photo-reversibility of the respiratory inhibition produced by carbon monoxide. A control curve is also shown in the figure.

Effect of added substrate and carbon monoxide on oxygen consumption by onion root tips: The results of the experiments presented in Table 3 had two main objectives; (a) to determine whether or not the tissue of the root tips would readily utilize glucose as a substrate, and (b) to establish the effect of carbon monoxide if glucose was utilized.

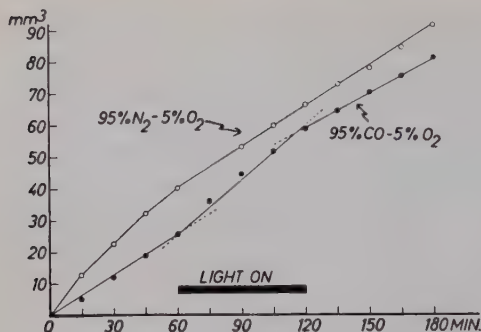


Figure 1. *Effect of carbon monoxide on respiration of onion root tips during alternate dark and light periods. On the ordinate mm³. O₂ consumed per 100 mg. (wet weight) tissue.*

Column I, Table 3, presents values for the oxygen consumed when the tissue was in an atmosphere of pure oxygen. These values were obtained to determine the maximum rate of respiration. Previous work (2) had shown that 50 % O₂ at a temperature of 30°C was sufficient to support the maximum rate of oxygen uptake. A comparison of values obtained in the control columns (II and VI — fresh sample) with those in Column I verifies this fact.

A comparison of the data yielded by fresh tissue samples indicates that 50 % CO causes no significant inhibition of oxygen consumption and that the respiratory rate of freshly cut root tips is essentially unaffected by the presence of glucose.

Based on the assumption that the failure of added glucose to bring about stimulation of the respiratory rate could be accounted for by the presence of adequate readily utilizable substrate in the freshly cut root tips, the experiments recorded in the lower portion of Table 3 were conducted. Roots were cut and the segments were allowed to remain in the mineral nutrient medium for approximately 15 hours prior to respiratory measurement. Three facts are now evident: (a) glucose stimulates oxygen consumption by about 34 % (b) carbon monoxide at this partial pressure causes significant inhibition, and (c) the endogenous rate of respiration in the starved roots is much reduced over that in the fresh samples.

Webster and Frenkel (29) have previously noted that the endogenous respiration of *Anabaena* was relatively insensitive to carbon monoxide until very high CO/O₂ ratios were employed, but that oxidation of substrate was strongly inhibited by carbon monoxide when the CO/O₂ ratio was lower.

Effect of added substrate and carbon monoxide on oxygen consumption by homogenate of onion root tips: The data in Table 4 were obtained using homogenates of root tips. The homogenate shows only about 4 % of the activity of the intact tissue; the oxygen uptake is 5 microliters per 100 mgs. per hour as compared to about 120 microliters for intact tissue. The data

Table 3. *Effect of added substrate and Carbon monoxide on oxygen consumption by onion root tips (0—5 mm.) (Cu. mm. of O₂ consumed/100 mg. (wet wt.) tissue/hr)*

Atmosphere				% Inhi- bition	Atmosphere			% Inhi- bition	% Stimulation shown by glucose
100 % O ₂	50 % O ₂ ; 50 % N ₂	50 % O ₂ ; 50 % CO			50 % O ₂ ; 50 % N ₂ + .1 ml. 0.1 M glucose	50 % O ₂ ; 50 % CO + 0.1 ml. 0.1 M glucose			
		Dark	Light			Dark	Light		
I	II	III	IV	V	VI	VII	VIII	IX	X
Fresh sample									
124	—	—	—	—	—	—	—	—	—
127	—	—	—	—	—	—	—	—	—
—	123	—	—	—	124	121 116	—	—	—
—	129 125	118	—	—	130	115	—	—	—
—	102	109	—	—	117	119	119	—	—
—	119	118	—	—	120	130	119	—	—
—	112	107	—	—	121	109	119	—	—
—	105	123	120	—	125	115	121	—	—
Average 117		115		1.7	123	118	120	4	5
Root segments starved 15 hours									
—	26.2	11.1	—	58	38.2	11.9	—	69	46
—	24.6	13.7	—	44	28.2	13.1	—	54	15
—	18.6	5.7	—	69	22.4	9.9	—	56	20
—	30	29	—	3	47	30	—	57	57
—	46.5	27.4	—	41	60	40	55	33	29
Average 29		17.4		40	39	21		46	34

in the table indicate that a gas mixture of 80 % CO—20 % O₂ fails to show significant inhibition in the absence of added substrate. The addition of glucose yields some stimulation of oxygen consumption, and carbon monoxide causes inhibition, which is photo-reversible. This is in accord with the earlier observations of Webster and Frenkel (29).

Effect of carbon monoxide on oxygen consumption by homogenate in the presence of cytochrome c and ascorbate: The results obtained upon the

Table 4. *Effect of added substrate and carbon monoxide on oxygen consumption by homogenate of onion root tips (0—5 mm. segments). (Cu. mm. of O₂ consumed/500 mg. (wet wt.) tissue homogenate/hr.)*

Atmosphere					
80 % N ₂ ; 20 % O ₂	80 % CO; 20 % O ₂		80 % N ₂ ; 20 % O ₂ + 0.1 ml. 0.1 M glucose	80 % CO; 20 % O ₂ + 0.1 ml. 0.1 M glucose	
	Dark	Light		Dark	Light
25	24	25	28	21	26

Table 5. *Effect of carbon monoxide on oxygen consumption by homogenate in the presence of cytochrome c and ascorbate.* (Cu. mm. of O₂ consumed/100 mg. (wet wt.) tissue homogenate/hr.)

(95 % N ₂ — 5 % O ₂)	Atmosphere			
	95 % CO — 5 % O ₂			
	Dark		Light	
	% Inhibition		% Inhibition	
107	4	97	35	67
99	0	100	23	77

Each vessel contained 0.3 ml. of ascorbate, 0.3 ml. of AlCl₃, 0.3 ml. of cytochrome c (10 mg./ml.) and 0.1 M phosphate buffer at pH of 7.4 to make a total of 3.0 ml. 0.2 ml. of KOH (15 %) was placed in the center well of each flask.

addition of cytochrome c and ascorbate, which is the usual method for a cytochrome oxidase assay, are recorded in Table 5.

The data show that oxygen consumption is now quite high (almost that of normal intact tissue), recalling that it was essentially negligible under these conditions in the absence of cytochrome c and ascorbate (Table 4). These data are corrected both for the blank uptake of oxygen in CO-O₂ mixtures and for the auto-oxidation of ascorbate.

The remaining data in Table 5 show that 95 % CO almost completely inhibits oxygen consumption and that this inhibition is partially reversible by light.

Discussion

The data presented in Tables 1 and 2, and Figure 1 leave no doubt that carbon monoxide inhibits oxygen consumption in the apical segment of the onion root tip, and furthermore that this inhibition is reversed by light.

Relative affinity constants of the affected enzyme for carbon monoxide and oxygen are not shown. Calculations by the older method of Warburg (27, see also 14, 28) show that for the data in Table 2 K_A values would range from 13 to well over 100. In intact tissue these values are not considered to be useful enough to merit inclusion for the reasons pointed out by Hartree (6, page 30).

Previous work has indicated a more marked and more easily obtainable inhibition when substrates were employed than when the inhibition was measured on the endogenous respiratory rate (29). It is of interest that with freshly excised onion root tips the addition of glucose has no effect upon respiratory rate; however a marked increase (34 %) was demonstrated if

the roots were excised and allowed to respire for some time prior to substrate addition and respiratory measurement. Even then the respiratory rate is not restored to anywhere near the original endogenous value. Also of interest is the fact that a CO/O_2 ratio of 1 causes no inhibition of the endogenous respiratory rate of freshly cut root tips, but causes marked inhibition of roots that have been excised and starved for 15 hours (Table 3). A study of the literature indicates that inhibition of plant respiration with a CO/O_2 ratio as low as 1 is quite unusual. The significance of the aforementioned observation is not readily apparent.

The endogenous respiratory rate of a homogenate of root tips is only about 4 % of the rate shown by intact tissue. A CO/O_2 ratio of 4 fails to show inhibition of the endogenous rate, but upon the addition of glucose there is a slight stimulation of oxygen consumption, and an indication that photoreversible inhibition is caused by carbon monoxide (Table 4).

The striking catalytic effect of added cytochrome c (together with an agent that can directly reduce it, ascorbate) is obvious from the data of Table 5. It would appear from these results that the cytochrome oxidase content of the root tip shows adequate activity to account for most, if not all, of the normal respiratory activity. Whether or not cytochrome oxidase does mediate most of the terminal hydrogen transfer in the normal respiration of this tissue is not established.

The presence of cytochrome oxidase in the onion root tip is established by (a) previously observed respiratory inhibition produced by cyanide and azide (1, 22) as well as the photoreversible inhibition of water absorption and bioelectric potentials by carbon monoxide (15, 21) and the spectrophotometric identification of cytochrome oxidase (11), (b) the photoreversible inhibition of oxygen consumption by carbon monoxide, and (c) the catalytic effect of added cytochrome c and ascorbate.

Part of this report was prepared during the summer of 1958, while the senior author was a National Science Foundation, Science Faculty Fellow, at the Marine Biological Laboratory, Woods Hole, Massachusetts.

Summary

The effect of carbon monoxide on the oxygen consumption of apical segments of the onion root has been determined employing conventional Warburg manometry. A CO/O_2 ratio of 19 shows inhibition averaging about 37 % as compared to values obtained in a control atmosphere of nitrogen and oxygen ($\text{N/O}_2=19$). This inhibition is photo-reversible.

A CO/O_2 ratio of 1 does not show inhibition of respiration of freshly

excised root tips, nor does the addition of glucose to freshly excised root tips raise the rate of respiration. If root tips are excised and then starved for 15 hours, an atmosphere of one half carbon monoxide and one half oxygen strongly inhibits the oxygen consumption both in the presence and absence of added substrate.

The addition of cytochrome c, along with an agent which can directly reduce it (ascorbate), to homogenates of onion root tips resulted in marked stimulation of oxygen consumption, which was demonstrated to be photo-reversibly inhibited by carbon monoxide.

These results are discussed, and it is concluded that cytochrome oxidase is present in the onion root tip, in amounts probably capable of mediating most of the terminal hydrogen transfer. No demonstration was made as to whether cytochrome oxidase functions as the terminal oxidase in the normal respiration of this tissue.

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Effects of Compounds of the Vitamin B₆ Group on the Growth of *Ophiostoma multiannulatum*

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It has previously been shown (Fries 1943) that the Ascomycete *Ophiostoma multiannulatum* is heterotrophic for vitamin B₆. The fungus has been used with success for the quantitative determination of this vitamin in natural substrates (Fries 1949 a). Of the three forms of the vitamin, pyridoxine, pyridoxal and pyridoxamine, the effect of only the first-mentioned was investigated. In 1951 two pyridoxamine-requiring mutant strains of this fungus, W 233 and W 366, were obtained by UV irradiation of conidia. A renewed study of the vitamin B₆ requirement of the fungus was therefore indicated, but could not be undertaken until recently. The present report is an account of the growth-promoting activity of the three forms of vitamin B₆ as well as of the phosphorylated derivatives of pyridoxamine and pyridoxal for the mutant strains and for the wild type.

Materials and Methods

After isolation, preliminary identification, and some mating experiments (see below) the mutant strains W 233, W 366 and 36 substrains from f₁ of the latter were kept alive by re-inoculations every half year on complete agar medium, consisting of basal nutrient solution supplied with 2.5 per cent malt extract (Vitrum), 0.25 per cent yeast extract (Difco) and 1.5 per cent agar. The cultures were stored at 4°C. When re-identification of the strains was performed in May 1957 it was found that W 366 and several of its f₁-substrains did not show any response to

pyridoxamine, but gave good growth in minimal medium supplied with pyridoxine. This reversion to prototrophy is probably due to backmutation of the gene responsible for pyridoxamine heterotrophy. One of the stable substrains, W 366-10, was chosen for continued study on account of its good conidial growth in liquid medium in shaken tubes.

As to the other strain, W 233, it was not suitable for growth studies in shaken tubes on account of hyphal growth. Therefore a daughter mycelium, W 233-2, obtained from a mating experiment with the mother strain and wild type, "Andrus" 1(—), was used instead.

The wild type strain used was No 51, of the morphological type called "Star".

The quantitative studies were performed in shaken tubes with the method devised by Fries (Fries 1949 a). The fungus was thus cultivated in tubes shaken mechanically at 30°C, each containing a final volume of 10 ml. nutrient solution. The growth was followed turbidimetrically in the Åberg-Rodhe turbidimeter. The extinction values are expressed according to the formula $Z = (e_v - e_0) \times 10^3$, where $(e_v - e_0)$ is the difference between the extinction value of the culture tube during the experiment and that of the tube immediately after the inoculation. All cultures were run in triplicate. The nutrient solution used was Fries' modified medium 3 with the omission of pyridoxine. The compounds under study in the respective cases were supplied to the tubes aseptically after having been sterilized by filtration through sintered glass bacterial filters. Since the vitamin is inactivated by light, work was as far as possible performed in diffuse light.

The conidia for inoculation were produced in shaken Pyrex flasks (125 ml.), each containing 20 ml. of medium and 20 μ mol of pyridoxamine. After 2 days the conidia were washed twice and transferred into medium without pyridoxamine for one day in order to exhaust them of stored vitamin. Before inoculation they were washed twice with distilled water by centrifugation. The suspension was diluted to an extinction value of 0.48, which corresponds to about $25 \cdot 10^6$ log-phase conidia per ml. (For starved conidia the number is higher because the conidia in such cultures are smaller than in growing ones). This suspension was diluted 50 times for inoculation. The chemicals used were the following:

Pyridoxal hydrochloride, Merck & Co., Inc. Rahway, N.J.

Pyridoxamine dihydrochloride, Merck & Co., Inc. Rahway, N.J.

Pyridoxine, Hoffman-la Roche & Co., Basel.

Pyridoxal-5-phosphate, California Foundation for Biochemical Research.

Pyridoxamine-5-phosphate, California Foundation for Biochemical Research.

Genetic data

Though there was little reason to question the heritability of the character pyridoxamine-less, one of the mutant strains, W 366(+), was mated with wild type mycelium No. 56(—). Out of 60 isolated 1-ascospore mycelia 36 proved to be pyridoxamine-requiring. When 20 of the pyridoxamine-less substrains in f_1 were crossed again with No. 56(—) wild type and No. 69(+) wild type, the cross revealed that both the expected mating types had segregated in f_1 , viz. 8(+) and 12(—). The character is thus heritable and probably a gene mutation. No. W 233 was also combined with wild type, "Andrus" No. 1(—). Out of 29 isolated ascospores 13 proved to be mutants, responding to pyridoxamine, but not to pyridoxine.

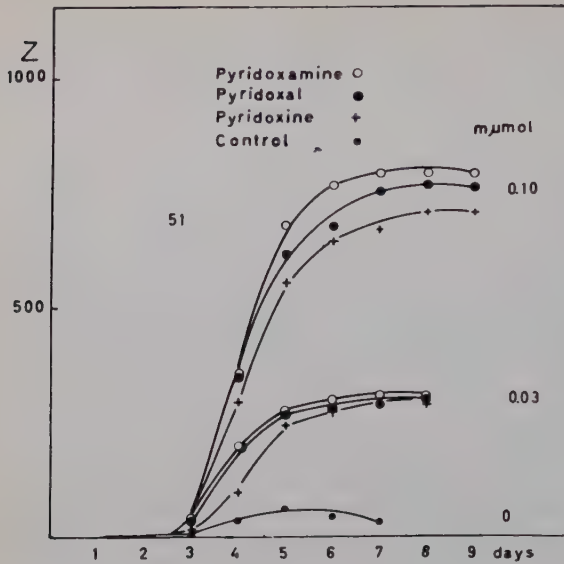


Figure 1. Growth (extinction values, Z) of No. 51, wild type, in shaken tube cultures with different amounts of pyridoxine, pyridoxal or pyridoxamine.

The Effect of Pyridoxine, Pyridoxal, and Pyridoxamine

Preliminary experiments. The identification of the mutants had been made auxanographically. These tests revealed the growth effect of pyridoxamine and the inhibitory action of pyridoxine and pyridoxal. Out of other available growth factors tested (including amino acids in acid hydrolyzed casein, nicotinamide, pantothenic acid, biotin, *p*-aminobenzoic acid, riboflavin, folic acid and B₁₂, and various nucleic acid constituents) only meso-inositol was active, growth being stimulated by it in combination with pyridoxamine.

Experiments with the wild type. As mentioned above previous studies with *O. multiannulatum*, wild type, concerning vitamin B₆ were performed with pyridoxine only. For a direct comparison between the response of the wild type and the mutants to pyridoxine, pyridoxal and pyridoxamine, the quantitative effect of these compounds was studied for the wild type, too. In several experiments the growth of No. 51, wild type, was followed by the turbidimetric method, the tubes being supplied with limiting amounts of pyridoxine, pyridoxal or pyridoxamine. Figure 1 shows the results of one experiment. It demonstrates the effect of the two lowest concentrations used in this experiment, viz. 0.03 and 0.10 μmol vitamin per tube. As is seen all three forms of the vitamin support growth. The amount of conidia produced depends on the amount of the vitamin given. As appears from the curves the final extinction values are nearly equal for each compound. On a molar basis the three vitamin analogues therefore seem to be equally active. The

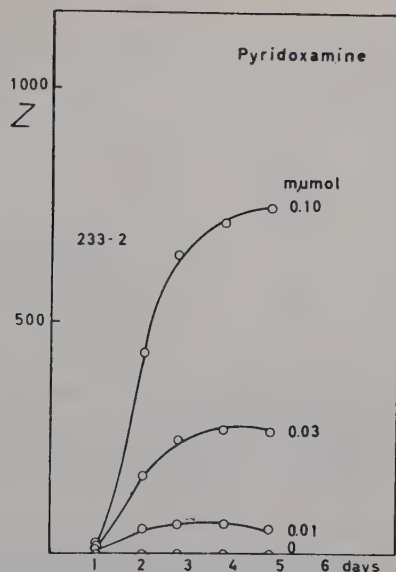


Figure 2. Growth (extinction values, Z) of No. W 233-2, pyridoxamine-less, in shaken tube cultures with different amounts of pyridoxamine.

similarity in form and time lag of the curves shows that the analogous are utilized at about the same rate. It must be mentioned, however, that the order of activity was always the same as above in all experiments performed, pyridoxamine giving the highest production followed by pyridoxal and pyridoxine.

Experiments with the mutants. In order to investigate the possible effect of pyridoxine on the mutants W 233-2 and W 366-10, this vitamin was tested for both mutants in the following concentrations: 0.01, 0.03, 0.10, 0.30, 1, 3, 10, 30, and 100 μmol per tube. Growth was not obtained with pyridoxine for any of the two mutants. It can thus be inferred that under the conditions used neither W 233-2 nor W 366-10 can utilize pyridoxine alone.

As to pyridoxamine the experience that this vitamin exerted a positive effect for the mutants in auxanograms was confirmed in quantitative studies for both mutants. Figure 2 illustrates the effect on growth for W 233-2 of pyridoxamine when cultivated with 0.01, 0.03 and 0.10 μmol per tube. Curves for higher concentrations are omitted. A comparison of the growth curves in Figure 2 with the corresponding curves in Figure 1 for the wild type shows that the mutant W 233-2 reacts quantitatively in about the same way for pyridoxamine as does the wild type.

In some experiments with the wild type a somewhat higher production was obtained than that indicated in Figure 1. It is therefore possible that the wild type may utilize the vitamin more effectively than the mutants are able

Table 1. *Effect of pyridoxal on the growth (extinction values, Z) of W 366-10 and W 233-2, pyridoxamine-less, in shaken tube cultures. Z-values at 4 to 7 and 2 to 7 days respectively.*

Pyridoxal μmol	W 366—10				W 233—2					
	4	5	6	7	2	3	4	5	6	7
0.10	0	0	0	0	0	0	0	0	0	0
0.30	0	0	0	0	0	0	0	0	0	0
1	0	0	+	+	0	0	0	0	0	+
3	+	158	621	840	0	0	0	70	221	320
10	59	477	745	760	0	0	16	100	290	480
30	246	1,000	1,343	1,350	0	60	145	467	930	1,187
100	400	930	1,300	1,361	35	255	467	925	1,316	1,366

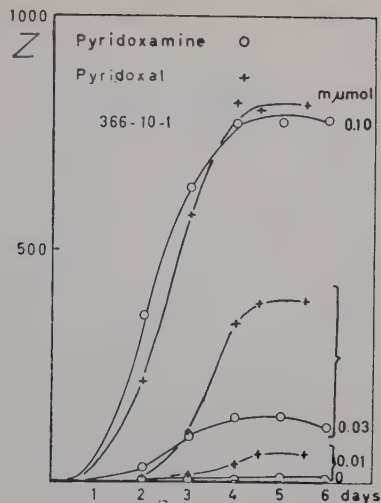
to. The response of the other mutant, W 366-10, for this vitamin was identical with that of W 233-2.

Pyridoxal was also tested for both mutants in different concentrations in shaken tube cultures. The amounts of the vitamin used and the results obtained are shown in table 1. As is seen growth is initiated for both mutants at the same concentration level, 3 μmol per tube, after a lag of 4—5 days. The lag is shorter when the concentration of the vitamin is increased. There is thus no difference in growth reaction between W 233-2 and W 366-10 with respect to pyridoxal under these conditions. If the results are compared with those obtained with the wild type it is clear that far higher amounts of pyridoxal are required to initiate growth for the mutants. For the wild type no lower concentration limit could be observed, below which pyridoxal was not active in the growth experiments. Nor could any noteworthy differences in growth lag be registered for different concentrations of the vitamin.

Some more information concerning the response to pyridoxal of the mutants was obtained from experiments to study the interaction between pyridoxamine and pyridoxal. An account of these is given below.

In order to study the possibility that conidia of W 366-10 might adapt themselves to grow on low amounts of pyridoxal or pyridoxine when previously raised on pyridoxal, a conidial suspension grown in a tube supplied with pyridoxamine 0.10 μmol +pyridoxal 30 μmol was plated out on minimal agar medium supplied with 1 μmol pyridoxamine per plate. Monoclonical mycelia were isolated on complete medium. One of these isolates named W 366-10-1 was analyzed in shaken tubes regarding its reaction pattern for pyridoxine, pyridoxal, and pyridoxamine. The inoculum was prepared from conidia grown in medium supplied with pyridoxamine. Pyridoxine did not support growth within 9 days tested in amounts from 0.1 to 10 μmol per tube. Figure 3 illustrates some growth curves obtained when limiting amounts of pyridoxal or pyridoxamine were present. Higher concentration

Figure 3. Growth (extinction values, Z) of No. 366-10-1, pyridoxal (pyridoxamine)-less in shaken tube cultures with different amounts of pyridoxal or pyridoxamine.



series (up to 10 μmol per tube) were omitted but gave a correspondingly higher production. From these curves it appears that W 366-10-1 responds to pyridoxal both quantitatively and qualitatively in about the same way as to pyridoxamine. At the lower levels pyridoxal produced more than did equal amounts of pyridoxamine. So far it is not known if W 366-10-1 represents a stable modification of W 366-10. W 233-2 has not yet been studied in respect to its possible capacity for adaptation.

It is thus evident that the adapted "strain" W 366-10-1 represents an intermediate type between No 51, wild type, on one side and W 233-2 and non-adapted 366-10 on the other. It responds to equimolar amounts of pyridoxal and pyridoxamine in about the same way as does No 51 to pyridoxine, pyridoxal and pyridoxamine, while W 233-2 and W 366-10 only respond to pyridoxamine and not to pyridoxal in the same concentration ranges.

At present nothing is known about the background of the difference in reaction pattern between W 366-10 and W 366-10-1.

The Effect of Pyridoxal Phosphate and Pyridoxamine Phosphate

The phosphorylated derivatives of pyridoxal and pyridoxamine must be looked upon as end products in the metabolism of vitamin B₆. In order to obtain information as to the effects of these compounds on *Ophiostoma*, they were tested in growth experiments with both the wild type and the mutants. Figure 4 and 5 show some of the growth curves obtained for No 51, wild type, when these substances were present in different limiting amounts.

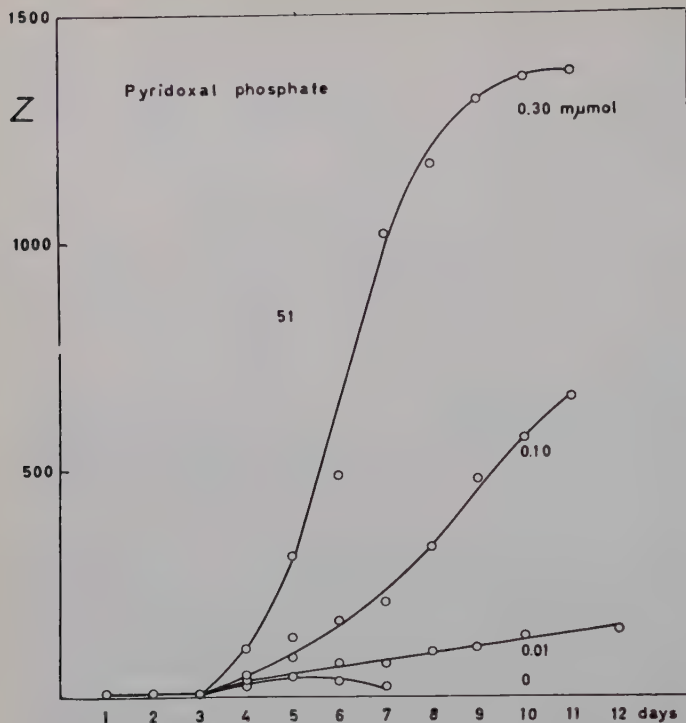


Figure 4. Growth (extinction values, Z) of No. 51, wild type, in shaken tube cultures with different amounts of pyridoxal phosphate.

As is clear from the curves both vitamin phosphates exert a positive effect in the same concentration ranges as do their unphosphorylated counterparts. The courses of the curves deviate, however, in two respects from those obtained with pyridoxine, pyridoxal, and pyridoxamine for the same object. The time lag is about one day longer as can be seen from a comparison with Figure 1, the results of which were obtained from the same experiment. Furthermore, the slopes of the curves for the two lowest concentrations reported are less steep owing to lower rate of growth during these experimental conditions. In some experiments erratic responses could be noted for the vitamin phosphates when present in the amounts 0.01 and 0.03 μmol per tube in so far that growth did not start at all in some of the tubes.

From experiments with the mutants it was found that pyridoxal phosphate and pyridoxamine phosphate also supported growth for these strains (Table 2). Some traits in the response to the phosphates can be observed from the values in the table. Higher concentrations were needed to initiate growth in the mutants than for the wild type. Below a certain concentration limit, 1 μmol for pyridoxamine phosphate and 10 μmol for pyridoxal

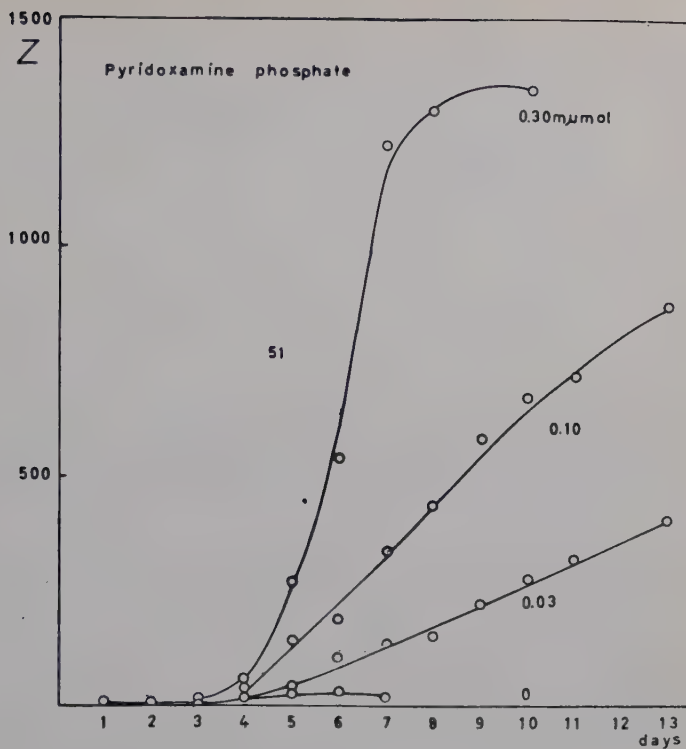


Figure 5. Growth (extinction values, Z) of No. 51, wild type, in shaken tube cultures with different amounts of pyridoxamine phosphate.

phosphate, no visible growth appeared. At the concentration limit a lag of variable length was obtained.

Summing up the results obtained in cultivating *Ophiostoma* wild type and the mutants with pyridoxal phosphate and pyridoxamine phosphate the following characteristic features as to the responses can be noted. In the wild type the rate of growth is lower for small doses and the growth lag longer at all concentrations tested than for the corresponding unphosphorylated vitamins. In the mutants the "minimal active dose" is about 100 times higher (1 μmol) for pyridoxamine phosphate and 1000 times higher (10 μmol) for pyridoxal phosphate than it is for pyridoxamine (0.01 μmol). The effect of the intact molecules of the vitamin phosphates on the mutants is difficult to evaluate since pyridoxal, which itself is active in about the same concentration as pyridoxal phosphate, or pyridoxamine may be responsible for the growth obtained on account of hydrolysis of the phosphates at the surface of the cells (see below).

All these findings seem to indicate that the utilization of the vitamin phosphates is impaired in comparison with the unphosphorylated vitamins.

Table 2. *Effect of pyridoxamine phosphate and pyridoxal phosphate on the growth (extinction values, Z) of W 366-10 and 233-2 in shaken tube cultures. Z-values at 2 to 6 and 2—8 days respectively.*

Addition μ mol	W 233—2					W 366—10					
	2	3	4	5	6	2	3	4	5	6	7
Pyridoxaminephosphate 0.3	0	0	0	0	0	0	0	0	0	0	0
1	0	39	270	1,280	—	0	0	0	30	206	455
3	45	1,096	—	—	—	190	1,078	—	—	—	—
Pyridoxal phosphate 3	0	0	0	0	0	0	0	0	0	0	0
10	0	0	19	240	950	0	0	0	0	100	914
30	—	268	1,273	—	—	35	906	—	—	—	—

It is possible that the effects can be attributed to difficulties for the phosphates to penetrate the cell membranes. Certain results of investigations in other fields seem to support this view. Thus phosphorylated thiamine was not as effective as thiamine itself in stimulating decarboxylations by tissues from thiamine deficient animals (Peters 1937). Experience of the inability of thiamine monophosphate to enter into intact cells of baker's yeast is also reported. In addition thiamine monophosphate was rapidly hydrolyzed by suspensions of cells from baker's yeast (Kiessling 1957).

In order to test if pyridoxamine phosphate could be hydrolyzed by cell suspensions of *Ophiostoma* the following experiment was arranged. A dense suspension (c. 5 mg. dry weight per ml.) of washed wild type conidia from a 2 day old culture was prepared in autoclaved $1/15$ M phosphate buffer, pH 6.0. The suspension was pipetted into two culture tubes, one of which was heated for 5 min. at 100°C . Pyridoxamine phosphate was supplied to the tubes (final concentration c. 1 mg. per ml.). During the experiment the tubes were shaken at room temperature. At intervals samples were taken from each tube and centrifuged. 50 μ l amounts of the supernate were chromatographed on Whatman No. 1 paper with the ascending technique according to the method of Fasella and Baglioni (1956). The solvent used was *n*-propanol and 10 % formic acid in the proportions 4 : 1. A control sample from pyridoxamine phosphate in buffer solution without cells was also run on the same paper. The procedure used allows good separation of pyridoxamine phosphate from pyridoxamine. After the completed run the spots were localized in UV light. The pyridoxamine phosphate spots were eluted with 0.1 N HCl and the amounts determined quantitatively in the Beckman spectrophotometer at 293 $\text{m}\mu$.

From the inspection of the chromatograms in UV it became clear that already 10 min. after the beginning of the experiment pyridoxamine had been formed in the tube containing the living cells. The fluorescence of this spot grew in intensity in the samples taken later, whereas the pyridoxamine phosphate spot showed a corresponding decrease. In samples from the suspension with the heated cells and in those from the tube with buffer without

Table 3. *Pyridoxamine phosphate treated with suspensions of living and dead cells of wild type Ophiostoma*. Pyridoxamine phosphate in μg . per 50 μl .

Samples taken after min.	Pyridoxamine phosphate from suspensions of	
	living cells	dead cells
10	50	51.5
55	40.5	51.5
110	15	50
245	5	49

cells, no pyridoxamine was found. The quantitative data for pyridoxamine phosphate are given in Table 3.

For the pyridoxamine spots no quantitative determinations were made but the identity of the compound formed with pyridoxamine was established by eluting a spot and rechromatographing the eluate with pyridoxamine as reference.

Cells from wild type *Ophiostoma* are thus able to hydrolyze pyridoxamine phosphate to pyridoxamine and presumably also pyridoxal phosphate to pyridoxal.

Interactions between Pyridoxamine and Pyridoxine (Pyridoxal)

As previously mentioned the auxanographic test in the identification procedure of the mutants had revealed an inhibitory action of pyridoxine and pyridoxal. In order to get some insight into these effects the interaction between pyridoxine and pyridoxal on one hand and pyridoxamine on the other was studied quantitatively. The growth curves of Figure 6 illustrate the results from an experiment with W 233-2, in which the tubes were supplied with 0.10 $\text{m}\mu\text{mol}$ pyridoxamine together with varying amounts of pyridoxine. Pyridoxine demonstrated its inhibitory effect on growth in two ways. The length of the lag phase of the curves was a function of the concentration of pyridoxine, being longer when the concentration of the vitamin increased. At 3 $\text{m}\mu\text{mol}$ pyridoxine the final extinction value was depressed, and 10 $\text{m}\mu\text{mol}$ per tube did not permit growth to start at all. Yet this amount did not kill the conidia. If a sufficient amount of pyridoxamine was supplied growth could be initiated again. — At 1 $\text{m}\mu\text{mol}$ pyridoxine (or less, not shown in Figure 6) the final production reached that of the tubes supplied only with pyridoxamine but did not exceed it. Thus pyridoxine cannot be utilized at all by W 233-2 under these experimental conditions. The effect of pyridoxal was not quite similar (Figure 7). In this experiment pyridoxamine 0.10 $\text{m}\mu\text{mol}$ per tube was combined with pyridoxal in different

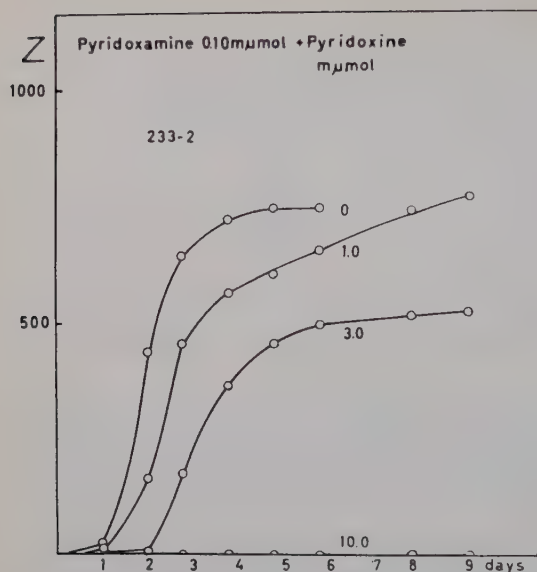


Figure 6. Growth (extinction values, *Z*) of No. W 233-2, pyridoxamine-less in shaken tube cultures with different mixtures of pyridoxamine and pyridoxine.

amounts. Pyridoxal revealed its inhibitory action by elongating the lag phase, but contrary to the combination pyridoxamine-pyridoxine the final growth values exceeded those of the tubes with pyridoxamine only, and at the level 30 μmol per tube the turbidity reached immeasurable values. Pyridoxal could thus in corroboration with previous evidence be utilized by W 233-2 to a certain degree, giving good growth in higher concentrations.

W 366-10 was also studied respecting its reaction towards mixtures of pyridoxamine and pyridoxine. Its reaction pattern under these conditions did not deviate from that of W 233-2, however. — With the combination pyridoxamine-pyridoxal the response varied somewhat. Inhibition by pyridoxal was always noted. In the first experiments pyridoxal could not, however, be utilized at all below 10 μmol. At this level the compound supported good growth after a lag of 3—4 days. Repeating the experiment half a year later with the same strain the reaction pattern of 366-10 resembled that of W 233-2 (Figure 7), pyridoxal being assimilated also at lower levels.

The above experiments with pyridoxamine in combination with pyridoxine and pyridoxal, respectively, indicated a competition between inhibitor and active vitamin. The phenomenon was studied in more detail for W 233-2 with pyridoxine as inhibitor. In two experimental series pyridoxine and pyridoxamine were supplied to the tubes in the proportions 10 : 1 and 30 : 1, respectively, over a 1000 fold range of concentrations. The concentrations used and the results obtained are seen in Table 4.

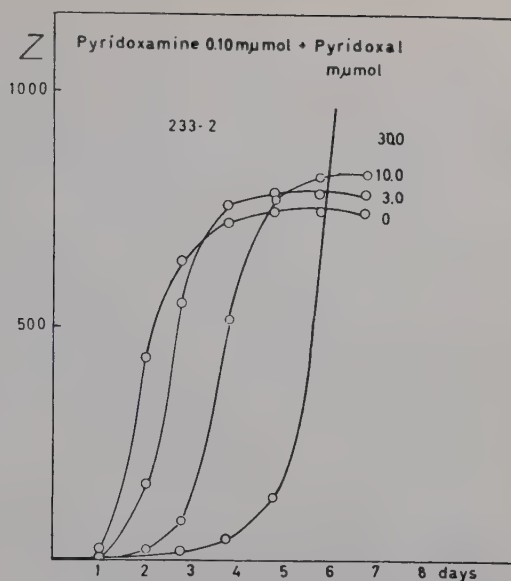


Figure 7. Growth (extinction values, Z) of No. W 233-2, pyridoxamine-less in shaken tube cultures with different mixtures of pyridoxamine and pyridoxal.

The results clearly show that the inhibition is competitive. A certain change in the concentration of the inhibitor requires a proportional change in the concentration of the active metabolite in order to produce the same effect. The inhibitory ratio for total inhibition was 100 : 1.

An experiment was made to determine the possible inhibitory action of pyridoxine for the mutant in combination with pyridoxaminephosphate and pyridoxal phosphate. Inhibition was found to occur, but the ratio between pyridoxine and the phosphates was in both cases 0.3 for total inhibition. This value is c. 300 times lower than that obtained with pyridoxamine.

Table 4. The inhibitory effect of pyridoxine on the growth (as Z-values) of the pyridoxamine-requiring strain W 233-2.

Pyridoxine μmol per tube	Pyridoxamine μmol per tube	Days of incubation		Ratio Pyridoxine/Pyridoxamine
		2	4	
3,000	300	1,000	—	10:1
300	30	750	—	
30	3	1,025	—	
3	0.3	1,060	—	
3,000	100	0	90	30:1
300	10	0	90	
30	1	0	110	
3	0.1	0	60	

Discussion

Vitamin B₆-requiring strains were among the first biochemical mutations obtained in *Neurospora sitophila* (Beadle *et al.* 1941). Later Stokes *et al.* (1943) obtained such mutants from *Neurospora crassa*. Some of these strains are able to synthesize the vitamin above pH 5.8 with ammonium ions in the medium. Others are pH-nonsensitive. The *Neurospora* mutants respond to all three forms of vitamin B₆. In spite of the fact that several thousand biochemical mutations have been isolated from the normally pyridoxine heterotrophic *Ophiostoma* the two strains now reported, W 233 and W 366, are the only biochemical mutants so far isolated which cannot utilize pyridoxine. When these strains were cultivated on the complete medium generally used in isolation experiments (Fries 1948) qualitative observations showed that growth was impaired in this medium as compared with minimal medium supplied with pyridoxamine. Both strains were obtained from a medium containing c. 10 % of the usual amount of malt extract, yeast extract and acid hydrolyzed casein (Wikberg and Fries 1952). It is therefore reasonable to suppose that the rare occurrence of this mutant type is due to inhibited germination of the mutated conidia. Apart from pyridoxine itself, which is shown above to inhibit growth, still other substances with the same property may exist in the complete medium. The same supposition has been suggested by Fries (1949b) to explain the rarity of guanineless strains in *Ophiostoma*. These are inhibited by hypoxanthine and adenine. Later it was shown (Fries 1950) that in isolation experiments guanineless strains occurred more frequently on medium supplied with guanine than on complete medium.

A situation of this kind is also known in histidinless mutants of *Neurospora crassa*. These mutants are inhibited by various amino acids in complete medium and have all been obtained from minimal medium supplied with histidin (Lein, Mitchell and Houlahan 1948, Haas, Mitchell, Ames and Mitchell 1952).

Summing up the observations made in this paper the following can be said regarding the responses of the respective strains.

No 51 grows equally well on both pyridoxine, pyridoxal and pyridoxamine. On molar basis the three forms are equally active. W 233-2 and W 366-10 do not respond to pyridoxine under any conditions tested. Pyridoxamine on the other hand supports good growth for both mutants and is equally active as pyridoxine is for the wild type. Pyridoxal is assimilated by the mutants but higher concentrations than for the wild type are needed to initiate growth (1—3 μ mol or more pyridoxal for the mutants, 0.01 μ mol for the wild type), and growth starts after a long lag.

One of the mutants, W 366-10, was able to adapt to pyridoxal response.

The adapted strain W 366-10-1 responds to pyridoxal and pyridoxamine in the same concentration ranges as does the wild type. Pyridoxine is not assimilated.

Pyridoxal phosphate and pyridoxamine phosphate support growth for the mutants but only at about 10 μ mol per tube for pyridoxal phosphate and 1 μ mol per tube for pyridoxamine phosphate.

For the wild type the vitamin phosphates exert activity in the same amounts as do the unphosphorylated forms, but the lag phase is lengthened and (in smaller amounts) the rate of growth is depressed.

The effects of pyridoxal phosphate and pyridoxamine phosphate may be interpreted in terms of permeation difficulties. In addition there is a possibility that the phosphates are hydrolyzed prior to absorption. Pyridoxamine phosphate is shown to be hydrolyzed to pyridoxamine by cell suspensions of wild type *Ophiostoma*.

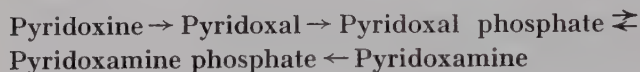
On account of the growth behaviour towards pyridoxine, pyridoxal and pyridoxamine of the strains studied it can be inferred that No 51 wild type is pyridoxine (pyridoxal, pyridoxamine)-heterotrophic, W 366-10-1 is pyridoxal (pyridoxamine)-heterotrophic, and W 233-2 and W 366-10 are pyridoxamine-heterotrophic. The heterotrophic characters of W 233-2 and W 366-10 have been shown to be inheritable and thus (gene) mutations. Intercrossing between the strains has not yet been undertaken. It is therefore not known if the strains are genetically identical or not. A genetic analysis of the strains is needed.

From studies of other biochemical mutations in microorganisms it is known that the effects on growth of structurally and physiologically related substances of the kind reported here may reflect the course of synthesis of the compounds within the cells, the biosynthetic path being blocked at different steps by mutations. From the viewpoint of biosynthesis pyridoxal phosphate and pyridoxamine phosphate, which have been shown to act as catalysts in numerous biochemical reactions, must be looked upon as end products in the metabolism of vitamin B₆, and pyridoxine, pyridoxal and pyridoxamine as precursors to the phosphates.

As to the wild type of *Ophiostoma*, the synthesis of the vitamin must be completely blocked before pyridoxine, pyridoxal and pyridoxamine, since B₆ must always be supplied to the medium for growth to occur. Contrary to the wild type the mutants described are not able to utilize pyridoxine. A genetic block must therefore be assumed to exist in the mutants after pyridoxine. Since one of the mutant strains, W 366-10-1, responds to pyridoxal and pyridoxamine but not to pyridoxine, pyridoxine presumably precedes pyridoxal and pyridoxamine, and since W 233-2 and W 366-10 show good response to pyridoxamine, while pyridoxal is less active, an incomplete block

in the synthesis of the coenzymes probably is situated after pyridoxal. Upon these assumptions pyridoxal is the second step in the biosynthetic sequence.

Though conclusions concerning the position of pyridoxal phosphate and pyridoxamine phosphate in relation to pyridoxamine and pyridoxal cannot be drawn owing to secondary growth effects of the phosphates, the following tentative biosynthetic scheme appears the most straightforward. The conversion of pyridoxal phosphate to pyridoxamine phosphate is assumed to be reversible.



From biochemical investigations it is known that both pyridoxine, pyridoxal and pyridoxamine are converted *in vivo* into codecarboxylase, which is identical with pyridoxal-5-phosphate (Bellamy, Umbreit and Gunsalus 1945, Trufanov and Kirsanova 1946). Not much information about the exact reaction sequence can be obtained from this field. Two enzyme systems related to the synthesis of the B₆-phosphates have been studied. Pyridoxal kinase has been isolated from yeast (Hurwitz 1953). The enzyme is, however, not specific for pyridoxal *in vitro*, since both pyridoxine and pyridoxamine are also phosphorylated (Hurwitz 1952). The other enzyme system, pyridoxamine phosphate transaminase, was found in *E. coli*. It mediated the interconversion between pyridoxamine phosphate and pyridoxal phosphate (Beechey and Happold 1957).

If it is assumed that the sequence in the synthesis of the coenzymes is the same for most organisms additional support for some steps in the scheme given above may be found in investigations with other microorganisms requiring vitamin B₆. From a collection of such data of activities (Williams *et al.* 1950) it is seen that the organisms responding to the vitamin can be divided into three groups. The first group utilizes pyridoxine, pyridoxal and pyridoxamine. It comprises various species of *Saccharomyces*, biochemical mutants of *Neurospora crassa* and *sitophila* and *Ophiostoma (Ceratostomella) ulmi*. Investigations by Fries (1943) showed two more species of *Ophiostoma*, viz. *O. fagi* and *piliferum*, to be pyridoxine-heterotrophic. The effect of pyridoxal and pyridoxamine for these two species has not been studied but in all cases reported in literature pyridoxine can be substituted by pyridoxal and pyridoxamine as growth factor. The two *Ophiostoma* species probably also belong to this group.

A characteristic feature for the second group of B₆-heterotrophies with an absolute demand for the vitamin is their inability to use pyridoxine while pyridoxal and pyridoxamine are assimilated. The organisms of this group

include species of *Streptococcus* and *Lactobacillus*, *Leuconostoc mesenteroides* and *Bacillus lactis acidii*. The growth requirements of these organisms may be explained by assuming a genetic block between pyridoxine and pyridoxal. Within this second group wide variations exist for different organisms in the ratio of the activities of pyridoxal and pyridoxamine. For one bacterial species pyridoxal may be more active than pyridoxamine, for another the circumstances are reversed. This may even hold for two strains of the same species. Thus in *Streptococcus lactis* the ratio of activity for pyridoxal-pyridoxamine is reported as 62.3 : 100 and 100 : 39.5 for two different strains. For *Lactobacillus casei* the same ratio is 100 : 0.7, this bacterium thus mainly responding to pyridoxal.

Even if other causes than (incomplete) genetic blocks may be responsible for the different relative activities of pyridoxal and pyridoxamine for different species of bacteria, the effects mentioned are in any case not in contradiction to the biosynthetic sequence proposed above. According to this scheme organisms may be imagined which respond only to pyridoxal or to pyridoxamine.

Finally two bacterial species represent a third group of B₆-heterotrophies. *Lactobacillus acidophilus* and *helveticus* grow only when pyridoxal phosphate or pyridoxamine phosphate is present in the medium. These two species may also fit into the proposed scheme if a double block is assumed, one before pyridoxal phosphate, and the other after pyridoxamine phosphate.

Antagonistic interactions between metabolites which act as growth factors for biochemical mutations are known to occur in several cases, *e.g.* in *Neurospora* for amino acids (Doermann 1944, Bonner 1946, Mathieson and Catchside 1955, Emerson 1949), and in *Ophiostoma* for nucleic acid constituents (Fries 1949 b).

The interactions described in this paper for the mutants when cultivated with the combinations pyridoxine-pyridoxamine and pyridoxal-pyridoxamine have been proved to be strictly competitive, when pyridoxine acts as inhibitor. The same probably holds for pyridoxal, but could not be shown for the strain used, W 233-2, since pyridoxal itself is assimilated to a certain degree. No inhibition could be noted for the wild type in amounts up to 10,000 μmol pyridoxine per tube. The fact that the antagonistic interactions have been revealed in cultivating the mutant strains does not, however, exclude the possibility that such a relationship also exists in the wild type, the phenomenon thus not being a direct consequence of the mutation to pyridoxamine-heterotrophy.

E. coli provides an example where the competition between valine and isoleucine was studied for both auxotrophic mutants and wild type. Growth of valineless and isoleucineless strains was competitively inhibited by iso-

leucine and valine respectively. On the other hand the incorporation of radioactive L-valine by *E. coli*, wild type, was inhibited by L-isoleucine when present in the medium (Cohen and Rickenberg 1956).

Summary

The Ascomycete *Ophiostoma multiannulatum* strain No. 51, wild type, and two (gene) mutations No. W 233-2 and No. W 366-10 of the fungus have been analyzed regarding their growth reactions towards pyridoxine, pyridoxal, pyridoxamine, pyridoxamine phosphate and pyridoxal phosphate.

No. 51 is pyridoxine (pyridoxal, pyridoxamine)-deficient. W 233-2 and W 366-10 are pyridoxamine-deficient but are able to utilize pyridoxal after a growth lag of 4—5 days at a concentration of about 100—300 times higher than the amount of pyridoxamine, which is required to initiate growth.

W 366-10 is able to adapt itself to grow at low levels of pyridoxal. For the adapted strain, W 366-10-1, the pattern of response is the same for pyridoxal and for pyridoxamine. This strain is thus pyridoxal (pyridoxamine)-deficient.

On the basis of the nutritional requirements of the strains a scheme for the biosynthesis of pyridoxal phosphate and pyridoxamine phosphate is proposed.

The effects on the growth of pyridoxal phosphate and pyridoxamine phosphate indicate difficulties in the utilization of these compounds. Rapid hydrolysis of pyridoxamine phosphate by cell suspensions of the fungus has been shown to occur.

Pyridoxine and pyridoxal have been shown to inhibit growth in the mutants by competing with the active form pyridoxamine. The ratio for total inhibition with the combination pyridoxine-pyridoxamine was about 100 : 1 for W 233-2.

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Respiration in Water Imbibing Barley Caryopses

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Ripe seeds germinate only when certain external and internal conditions are favorable, *i.e.*, the temperature must be suitable, the water content of the seeds must be above a certain level, and, furthermore, seeds of most species require access to oxygen. Under natural conditions the factor controlling the rate of germination is frequently the water content, since the intensity of the respiratory processes which provides the basis for the intensive manifestation of life during the initial growth of the embryo varies with the amount of water present in the seeds (see, *e.g.*, Bailey 1940).

However, the water content affects not only the intensity of the respiration, but also the mutual balance between aerobic and anaerobic processes, and hence affects the experimental possibilities of measuring the respiratory intensity by determining the gas exchange (uptake of O_2 or evolution of CO_2). In the case of more extensive respiration studies, where the aim is to determine the type of material respired, or to follow the course of the respiratory processes, it is not sufficient to measure the changes of O_2 or of CO_2 separately, but it is necessary to measure the intake or output of both gases simultaneously — as emphasized already by de Saussure (1804) — and to relate the gas volumes measured to each other, usually by computing the fraction $\frac{CO_2}{O_2}$. This quantity is usually termed the respiratory quotient, RQ.

However, since the changes in the amounts of gas measured are not always necessarily due to the respiratory processes alone since other metabolic pro-

cesses may interfere, the more neutral term of gas quotient, GQ, is usually preferable.

Godlewski (1882) appears to have been the first to show the hydration of seeds to be of importance for their GQ. Godlewski found air dried peas to have a GQ of 1 or very close to 1, whereas peas which had been placed in water for 48 hours showed values close to 2. Several later studies have confirmed these observations of a high value of the GQ in seeds containing water. Frietinger (1927) found for peas a maximum value of about 5. The greater values were found in experiments with unshelled peas, and in cases where the peas were immersed in water during the experiment. Frietinger's apparatus which used a closed system of circulating gas allowed changes of the rate of the gas stream to be made. Such changes which must cause changed conditions for the gas exchange of the seeds showed the GQ values to be most readily affected when the peas retained their shell and/or were immersed in water. As stressed by Geiger (1928) these results may be explained simply on the basis of the physical presuppositions, that the gas exchange in immersed and in water containing seeds is reduced by the slow diffusion of the gases through more or less stagnant layers of water. Furthermore, if the supply of oxygen is reduced anaerobic conditions may result in the seeds, the relation between CO_2 evolved and O_2 taken up then being disturbed by the more or less intensive anaerobic respiration.

A representative example of the complications which a diffusion inhibiting layer of water may introduce into respiration measurements is found in a work by Ruhland and Ramshorn (1938). They show a strong increase of the GQ in root tips of *Vicia faba* to take place upon the addition of a small excess of water, resulting from a reduction of the uptake of O_2 and an increase of the output of CO_2 .

The rates of diffusion of the two gases, oxygen and carbon dioxide, are not reduced to the same extent by the diffusion inhibiting layer due to the difference in solubility in water. The rate of diffusion of gases in water is proportional to their solubility, all other conditions being equal. Briggs and Robertson (1948) report the diffusion of CO_2 to exceed the diffusion of O_2 by a factor of about 26 through the same aqueous medium. In highly aqueous and possibly immersed tissues, as represented by seeds during imbibition, difficulties may therefore arise concerning the supply of O_2 , while the output of CO_2 is not similarly affected. Under such conditions measurements of the gas exchange will show the uptake of O_2 to be considerably reduced, while the output of CO_2 may be increased, partly because the CO_2 diffuses comparatively easily into the gas phase in which the measurements are made, and partly because water induced anaerobic conditions may cause a production of CO_2 without a corresponding uptake of O_2 .

Stålfelt (1926) also demonstrated this type of inhibition of the uptake of oxygen through layers of water, and showed that an increase in the pressure of O_2 could cause an increase of the uptake of O_2 in seeds of *Sinapis*.

In all of the above studies the plant material used had a constant water content, in fact it was probably saturated with water. James and James (1940) studied the changes of the GQ during uptake of water (varying water contents). They used seeds (caryopses) of barley and determined the exchange of gas during the first 48 hours after the plant material was moistened. The experiments show (James and James) the GQ to increase during the first few hours to values slightly above 1.5, and then to fluctuate and finally to approach a value of 0.95. According to James and James the high value of GQ is due to the imbibition, which as mentioned above changes the conditions of diffusion for the gases. In agreement with this fact the authors (1940) showed that while the output of CO_2 increases steadily and evenly the increase in the uptake of O_2 is uneven, hence the variations of the GQ values.

To elucidate further these phenomena the present author designed the following experiments on barley caryopses.

Material and Methods

Caryopses of barley was chosen for the study because with this plant material it is possible to vary the thickness of the layer of water or aqueous tissues through which the gases must diffuse in three ways: 1) By using caryopses with attached husks, 2) by using caryopses from which the husks are removed, and 3) by using isolated embryos. Caryopses of the variety "Carlsbergbyg" were used.

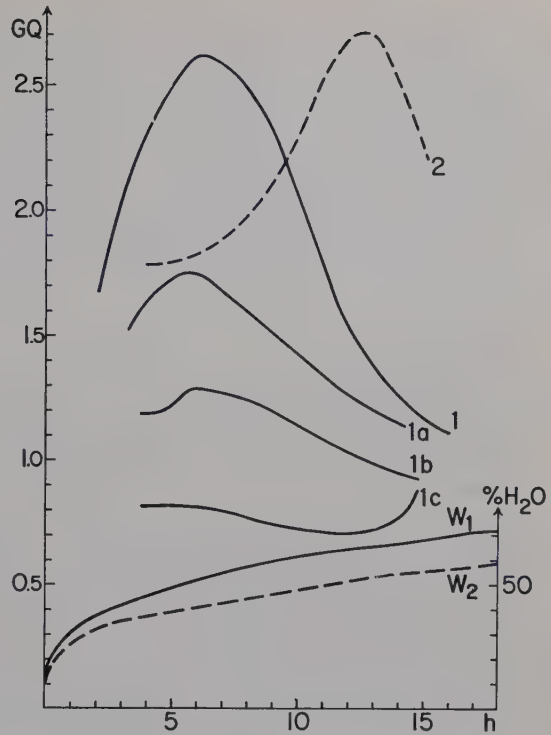
The gas exchange was measured by means of the "direct method" of Warburg as described by Umbreit, Burris and Stauffer (1951). The amount of water in the vessels was kept at a minimum, the consideration of the water uptake being partly counteracted by a consideration of the fact that a large amount of water may introduce experimental errors of the type demonstrated by Ruhland and Ramshorn. The sample vessels contained 10 or 20 caryopses or embryos depending upon the temperature. Manometer readings were made at 1 hour intervals.

Since the gas exchange is indeed assumed to vary with the imbibition, the water uptake of the barley caryopses was determined simultaneously with respiration experiments according to a method described by Allerup (1958). The removal of husks and the isolation of the embryos may, with a bit of practice, be done quite easily after the caryopses have been soaked in water for 1 to 1½ hours. The experiments were made at the temperatures of 27 and 17°C. The results shown are mean values of 2 to 3 experiments. No attempt was made to keep the plant material in the dark during the experiments.

Results and Discussion

Experiments at 27°C. It appears from Figure 1 that caryopses with husks (curve 1) show a distinct GQ maximum at about 6 hours after the time of

Figure 1. GQ-variation and water uptake in barley caryopses. Abscissa: time in hours from the moistening of the plant material. Ordinates: left GQ, right water content on a dry matter basis. Curve 1: GQ for caryopses with husks at 27°C.; curve 1 a: caryopses without husks at 27°; curve 1 b: isolated embryos at 27°; 1 c: caryopses without embryos (only aleuron cells and amylase cells alive) at 27°; curve 2: GQ for caryopses with husks at 17°C.; curve W₁ and W₂: water uptake in barley caryopses with husks at 27° and 17°, respectively.



moistening. The present GQ values are higher than the values found by James and James, probably due to the fact that different techniques were used for the two studies; in particular it appears to be reasonable to assume that in the present experiments the caryopses were covered by a layer of water thicker than that used in the experiments by James and James, and that generally speaking the possibilities of water uptake open to the plant material were different in the two studies. The curves for caryopses without husks (1 a) and for isolated embryos (1 b) run similarly but without attaining as high GQ values as curve 1, which may be explained by the fact that diffusion here proceeds through thinner layers of water and consequently causes less differentiation between O₂ and CO₂ diffusion.

It should be emphasized that the GQ maximum occurs at virtually the same time in caryopses with or without husks, and in isolated embryos. The gas quotient of the aleuron and amylase cells (all other parts of the endosperm is reported to consist of dead cells according to Lehmann and Aichele 1931, and to Müller and Holm 1942) remaining after the isolation of the embryo has been studied as well (curve 1 c). In this case the GQ varies in

an entirely different way, the values being nearly constant, about 0.8, during the first few hours, decreasing slightly only later, and finally rising to a value of 1.0. As appears from curves 1, 1 a, 1 b, and 1 c, the GQ maximum occurs only in cases where the plant material included embryos. Hence the GQ variation as it appears in curves 1, 1 a, and 1 b must be due to the respiration of the embryo for which conditions change as the imbibition proceeds from a phase, during which the water causes an increasing inhibition of the uptake of O_2 , to another phase during which the inhibition of the O_2 -uptake decreases as the tissues approach the natural state of high turgor with air-filled intercellular spaces.

During this change of phases it seems possible that the intercellular spaces for some time at least are filled with water. In this connection it is worth noting that Krogh (1919) showed oxygen to diffuse through animal tissues more slowly than through water, the diffusion constants (cm.^3 gas per minute through a 1μ membrane of an area of 1 cm.^2 at a difference in pressure of 1 atm.) being given as 0.34 for water, 0.14 for muscle and 0.115 for connective tissue. The animal tissues mentioned contain no intercellular spaces and may therefore be compared to compact seed tissues undergoing the first phase of imbibition. Even if the intercellular spaces of the fully imbibed plant tissues were filled with water the tissues could thus become more easily penetrable to O_2 as the imbibition proceeds.

In Figure 1 a curve for the water uptake in caryopses with husks at 27°C . (W_1) have been drawn using the same time scale as used for the GQ curves. The GQ maximum appears to occur at a water content of 50 to 55 per cent on a dry matter basis.

Experiments at 17°C . To further elucidate the connection between the GQ variation and the imbibition an experiment was made using caryopses with husks at 17° , as shown in the curve 2 of Figure 1. In this also a distinct GQ maximum appears, although at a later time corresponding to a lower rate of the water uptake at the lower temperature. By comparison with the water uptake curve for 17° (W_2) the GQ maximum is found here also at a water content of 50 to 55 per cent., on a dry matter basis.

Summary and Conclusions

Previous reports of high GQ values in seeds containing water, and of variations of the GQ in imbibing seeds are confirmed. The high GQ values may be explained by changes in the diffusion conditions for the gases O_2 and CO_2 when water penetrates into the seeds. In experiments with barley caryopses the conditions of the diffusion were changed by removing the

husks and by isolating the embryos, and also by varying the temperature of the experiments (27 and 17°C). In all cases the GQ maximum occurs at a time corresponding to a water content in the caryopses of 50 to 55 per cent., on a dry matter basis. The O₂-supply of the tissues may be transiently inhibited more than the output of CO₂, and the reduced oxygen supply may cause transient anaerobic conditions; however, this appears not to injure the embryo. Such periods of completely or partly anaerobic conditions may be assumed to occur during natural conditions of germination as well.

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Growth and Cell Division in the Apical Meristem of Wheat Roots

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The studies on the growth of wheat roots are very advanced due to the investigations of Burström and his collaborators (for literature see 6 and Lundegårdh 23). There is, however, one topic which has been comparatively neglected — the study of the gradients of growth and of cell division in the meristem.

The first question is: what is the longitudinal growth rate of cells in different parts of cell division zone? More recent studies on this problem in *Zea* and *Phleum* roots have been carried out by Erickson *et al* (12, 14) and Goodwin *et al* (15, 16). They have shown that the growth rate changes gradually along the whole growing zone of the root, also in the cell division zone where it increases from the tip.

The second question is connected with cell division rate: what is the frequency of cell formation in different parts of the meristem? Also this problem has been treated by the mentioned authors. They came to the conclusion that the rate of cell formation has a distinct maximum at about middle point of the zone. Since this change of cell formation rate is valid in respect to a single row of cells it follows that the duration of the over-all mitotic cycle of a cell (from one division to the next) varies with the distance from the tip. Therefore, one can also expect that the duration of a particular stage of mitotic cycle, like anaphase or metaphase, is different in different parts of the meristem. Thus the third question arises: what is the duration

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of the mitotic stages in different parts of root meristem? These three questions open the present study.

The present paper is based on the Goodwin-Erickson method (16, 13) for obtaining cell formation rates. This method is developed to give data on the duration of mitotic stages.

Terminology

- x = the distance from tip. The tip is defined here as the point of junction between the calyptrogen and the root meristem. (Dimension: mm.).
- dx/dt = rate of elongation of apical segment of root x mm. in length = rate of displacement from the tip of a point located at x . (Dimension: mm.hr⁻¹).
- dc/dx or N_c = cell density at x in a single file of cells. $dc/dx = 1/l$, where l = average length of cells at x . (Dimension: no. of cells · mm⁻¹).
- dc/dt = rate of cell formation by an apical segment of a file;¹ (Dimension: hr⁻¹). Calculated from dc/dx and dx/dt : $dc/dt = dx/dt \cdot dc/dx$.
- $d(dc/dt)/dx$ = frequency of cell formation at x ;¹ the slope of the tangent to the curve dc/dt . Calculated as the derivative of the function $dc/dt = f/x$. (Dimension: mm⁻¹ · hr⁻¹).
- $d(dc/dt)/dc$ = rate of cell formation per cell at x ;¹ (Dimension: hr⁻¹).
- N_m = density of cells in the particular stage of mitosis. (Dimension: no. of cells · mm⁻¹).
- T_c = duration of over-all cycle; the time between two divisions. It may be calculated from the cell formation rate: $T_c = 1/d(dc/dt)dc$. (Dimension: hr).
- $T(m)$ = duration of particular mitotic stage (adequate symbol may be put in the parenthesis, *e.g.* T_a — the duration of anaphase). (Dimension: hr). $T_m = N_m/d(dc/dt)/dx$. This formula derives from the fact that the relative number of cells in the given stage of mitosis is proportional to the fraction of the total time of over-all cycle that is taken up by this stage (Brown 4). $T_m/T_c = N_m/N_c$. Substituting $T_c = N_c/d(dc/dt)/dx$, one can obtain the mentioned formula.

Goodwin-Erickson method as well as the method for calculating the mitosis duration are applicable provided that: 1) the growth pattern and cellular pattern in the meristem remain constant, 2) the cells divide without any diurnal rhythm, 3) there is no difference in rate of division of cells at the given distance from the tip. It must be kept in mind that the pattern of growth and of cell division ought to be constant not only during the actual measuring of growth but also during at least the last preceding day, since the actual cell configuration is a result of the growth and the cell divisions which occurred in the preceding period.

In order to check the first condition a preliminary study on the develop-

¹ Only the transversal divisions, *i.e.* the increase in the number of cells along the root axis.

ment of *Triticum* roots has been made (this will be published elsewhere). This study showed that the pattern of growth undergoes some change in time even if constant and optimal environmental conditions are maintained, namely 1) the total growth rate decreases slowly after 6—7 days following germination, 2) the elongation zone as well as the cell division zone are slowly shortened. These changes, however, occur so slowly, at least for the first three days of growth in nutrient solution, that the deviation from a constant pattern may be neglected for the purpose of the method.

A preliminary count of mitoses in squashed root tips in acetone-grocin showed that there are no significant differences between three times of day: at 8 A.M., 4 P.M. and 12 P.M., which agrees with the statement of Brown (4) that in root meristems under constant environmental conditions divisions occur without any diurnal rhythm. So it appears that the number of mitoses found on any one occasion is representative of the period of time of the experiment.

The third condition is rather difficult to prove experimentally. The morphological difference between different layers of the meristem make us suspect that the condition is not fulfilled throughout the whole transversal section. It appears, however, that within any one morphologically uniform cell layer (at given distance from tip) there are no differences between the cells in the probability of their entering mitosis. But the different layers of the meristem were treated separately in the present investigation.

Material and Methods

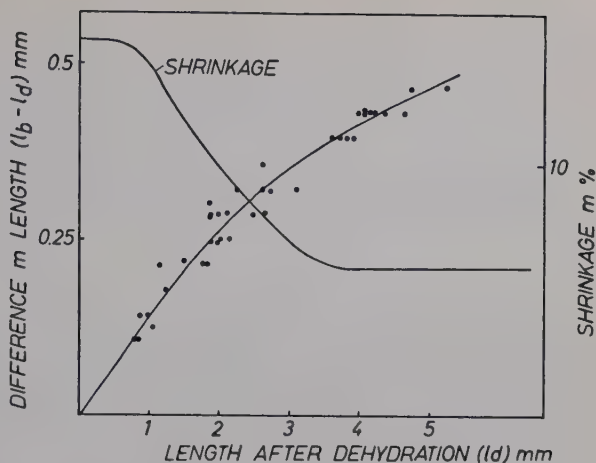
The present investigation have been performed with the primary and the first two lateral roots of intact seedlings of wheat (var. Weibull's Eroica).

Soaked seeds were germinated in the light on gauze stretched tightly over shallow dishes containing a little tap water. The dishes were placed in a large vessel covered with a glass plate and containing enough water so that the edges of the gauze dipped down into the water on all sides. With this method seedlings were obtained with fairly straight roots, not geotropically induced. On the second day after germination the seedlings were planted in the holder of a culture vessel, $20 \times 15 \times 2$ cm., made from transparent perspex (ten plants per vessel), and the vessels were placed in front of a daylight fluorescent tube (40 W).

The nutrient solution had the following composition: KNO_3 10^{-3} M, $\text{Ca}(\text{NO}_3)_2$ $2 \cdot 10^{-3}$ M, KH_2PO_4 10^{-3} M, Na_2HPO_4 $2.5 \cdot 10^{-4}$ M, MgSO_4 $5 \cdot 10^{-4}$ M, MnSO_4 $2 \cdot 10^{-5}$ M, Fe-versedate $2 \cdot 10^{-5}$ M. During the culture period it was aerated. The illumination and the temperature, $+18^\circ\text{C}$, remained constant during the course of experiment.

For this investigation two sets of data are needed, one from the living roots (distribution of growth) and the other from fixed and sectioned ones (cell length, distribution of mitoses). In order to make them comparable, the data from fixed

Figure 1. Difference in length of apical segments of root caused by fixation and dehydration (increasing curve) and calculated gradient of shrinkage in %.



roots must be corrected for the shrinkage caused by the fixation and following treatment.

For estimating the shrinkage the apical 1 to 6 mm. (excluding root cap) were cut off and the segments individually treated with the same procedure as the experimental ones (see below). The length of segments was recorded: 1) before fixation — l_b , 2) after fixation — l_f , 3) after dehydration — l_d . There was distinct difference between l_b and l_f or l_d but not between l_f and l_d . It means that nearly the whole shrinkage was caused by fixation. The results expressed as $l_b - l_d$ and plotted against l_d are given in Figure 1.

The shrinkage along the root axis evaluated by means of the first derivative of the function $l_b - l_d = f(x)$ against x (Figure 1), is rather constant in the first millimeter from the tip and then decreases gradually. To correct the length of the fixed roots the following values of shrinkage were accepted: 15 % (of length in the fixed material) in zone 0.0—1.00 mm., 14 % in zone 1.00—1.25 and 12.5 % in zone 1.25—1.40 mm.

Growth Distribution

The growth distribution has been studied by means of photographic records. A horizontal microscope with 10 \times objective and 6 \times ocular with camera was employed. The seedlings were allowed to grow under experimental conditions for 48 hours, and then 15 selected roots growing vertically were photographed on 36 mm. Adox KB 14 film. Beginning with the tip the surface of the apical parts of the roots was photographed with a sequence of about 3 overlapping pictures with an exposure time of 1 sec. An average time of 5—10 sec. elapsed between each exposure. Such a sequence was repeated for the same root at intervals of about 40 minutes accurately measured by means of a stopwatch. The roots were photographed alternately.

Photographic enlargements, 70 \times , were matched to reconstruct the root surface. There were always some characteristic points of the cell wall net present on all the photographic reconstructions of a given root. The displacement rate of these points was calculated by comparison of the reconstructions.

Since the root tip was not very clear, the displacement of the points was calculated with reference to the first clear point nearest the tip (usually about 0.2—0.3 mm. from it). The displacement of this reference point itself in respect to the root tip was estimated by comparing the photographs taken at intervals of about 4 hours. For other points in the apical region the displacement rate was obtained by comparing the pictures taken at intervals of about 40 min. The displacement rate calculated in mm. per hour was plotted against the mean distance of the point from the tip during the displacement.

Cell Length, Cell Number and Mitosis Distribution

Immediately following photographic records the apical parts, 5 mm. in length, of both the recorded and neighbouring roots were cut off and fixed in *Cr-A-F* solution (0.4—3—10). All the photographically recorded roots were treated individually. The dehydration and embedding were performed according to the standard tertiary butylalcohol-paraffin method.

The photographically recorded roots were cut transversally into 11 μ sections (corrected for shrinkage) for counting of the mitoses, and the other roots were cut longitudinally for measuring cell lengths. Both types of sections were stained by the neutral violet-iodine method and counterstained with saturated solution of Orange G in absolute alcohol.

The cell density was estimated on the central longitudinal sections of 15 roots by means of 10 \times objective and 8 \times ocular-micrometer divided in 10 scale units. The cells were counted in the apical 1.28 mm. of the root (corrected for the shrinkage). The number of cells per unit was estimated with an accuracy of $1/10$ — $1/5$ of cell length. Counts were made on the epidermis, the first two layers of cortex (separately) and the mother cells of the central metaxylem vessel. The average number of cells per scale unit was recalculated per 1 mm. and in this way cell density (number \cdot mm.⁻¹) for the segment has been estimated.

The counting of mitoses was performed on the same cell layers as cell density. The following stages were counted: 1) prometaphase and metaphase together — thick chromosomes, nucleoli absent; 2) anaphase — chromatids separated; 3) early telophase — chromosomes less distinct but the mass strongly stained, nucleoli absent, cell plate formation incomplete. Only mitoses leading to transversal cell division were counted. The series of transversal sections from 12 previously photographed roots were examined. On each section the stages in question were recorded after having been traced on the neighbouring sections to avoid duplicate counting.

In order to evaluate the linear density of mitoses the average number of the cells in a given stage present in the groups of 6 sections was recalculated per 1 mm.

The number of files in a given tissue or layer has been estimated as the number of cells which appear in the transverse sections.

Results

Growth Distribution

The principal attention has been paid to the growth of the apical part of the root (the first 2 mm.). There are, however, some data referring to the whole growing zone. The length of the growing zone is 4—5 mm. and the

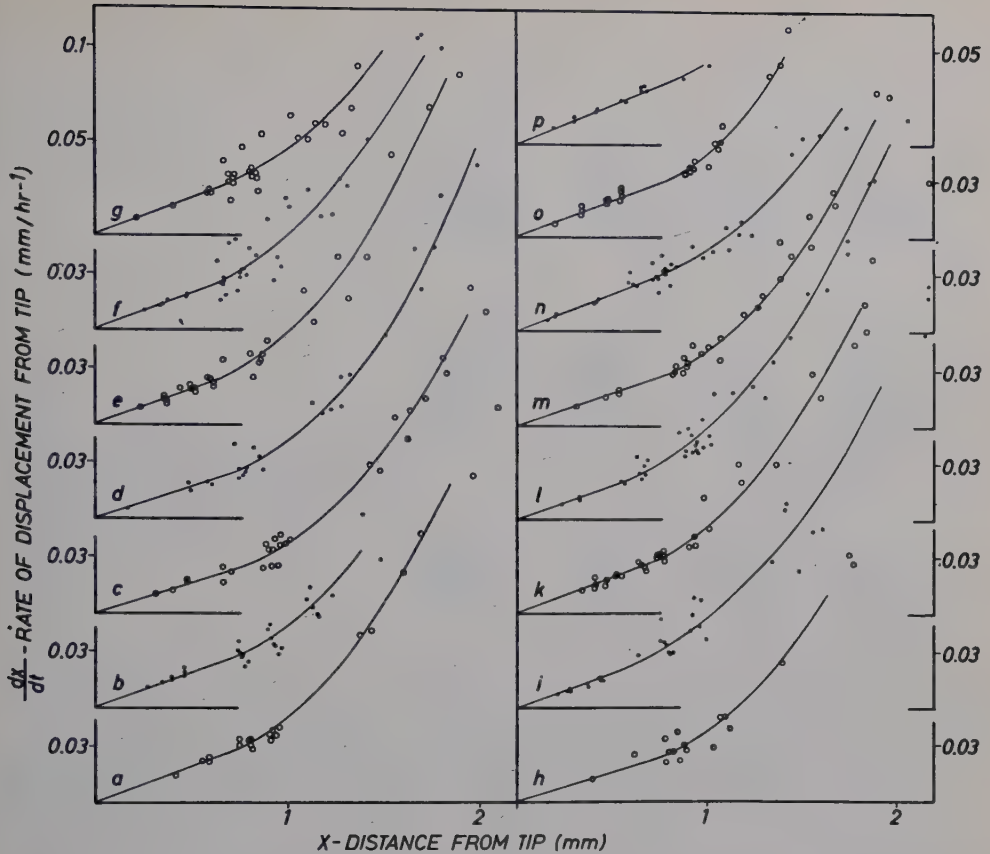


Figure 2. Rate of displacement of characteristic points of cell wall nets plotted against distance from the tip in apical part of growing zone of wheat roots. The curves have been displaced successively upward to avoid overlapping.

total growth rate amounts to 0.6 mm./hr. The detailed data on the growth in the apical part are given in Figure 2. As can be seen, the displacement rate in the region 0.0—0.8 mm. is fairly proportional to the distance from the tip. It means that the growth rate is constant in this zone. It amounts to the value 4.0 per cent. The different roots were very similar to each other in respect to the growth in the apical part of growing zone, so that it was easy to estimate the average values of the displacement rate in this part. These values were used in calculation of cell division rates.

Cell Density

The results of counting the cells recalculated as no. of cells per mm. of one file are presented in Figure 3. It may be seen that the cell density in the

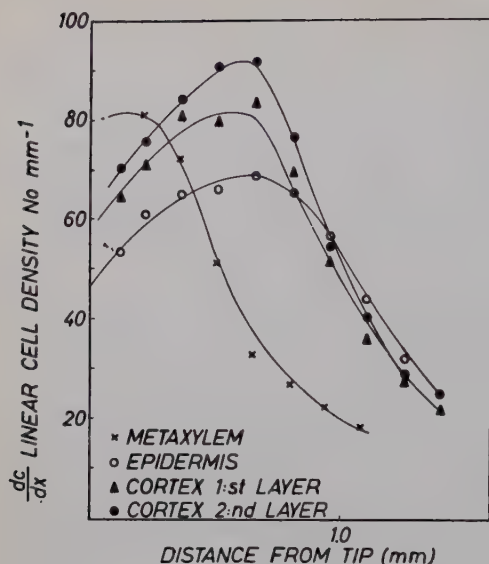


Figure 3. Linear cell density in a file of cells in some tissues of wheat root meristem, i.e. the number of cells in successive sections of a file computed per 1 mm.

epidermis as well as in the cortex is distinctly lower at the extreme apical part than it is about 0.6 mm. from the tip. Beyond 0.65 mm. the cell density progressively decreases. In the case of the metaxylem this decrease starts just after a point 0.2 mm. from the tip. As cell length is a reciprocal of the cell density in a file, the cell length first decreases with distance from the tip and then increases. That is in agreement with data on *Phleum* (15) and *Zea* (13, 19).

Distribution of Mitoses

The distribution of mitoses (from prometaphase to early telophase) in the epidermis and in the first layer of cortex in every root examined is depicted in Figure 4 (the second layer of cortex has a distribution similar to the first one). It may be seen that the region of maximum frequency of mitosis lies somewhat away from the tip. This agrees with the work of Wagner (27), Jensen (21) and Hussein and Heneen (20).

On the first two to three sections (from the tip) only a few mitoses were observed and they usually occurred in peripheral layers. That is in agreement with the data of Cloves (10, 11), who has demonstrated a metabolically inactive centre near the tip (see Discussion). Somewhat further from the tip mitoses start rapidly in all tissues and then they stop also rather quickly at the end of the cell division zone. The length of this zone varies in different tissues. It terminates closest to the tip in provascular tissue like metaxylem mother cells (0.4 mm. from tip). The length of the cell division zone is

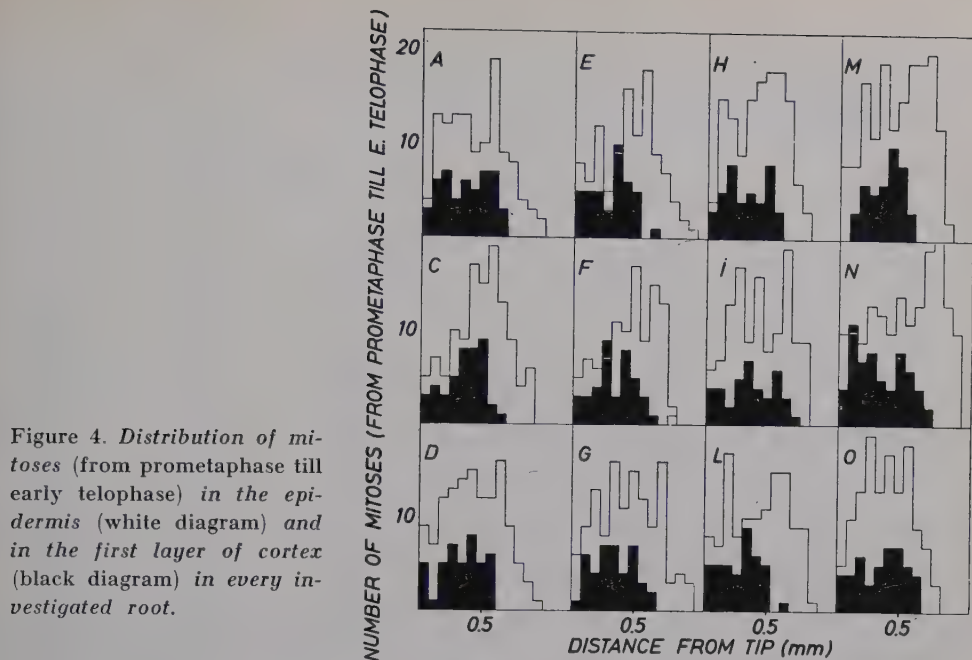


Figure 4. *Distribution of mitoses (from prometaphase till early telophase) in the epidermis (white diagram) and in the first layer of cortex (black diagram) in every investigated root.*

identical in the first two layers of cortex, but it seems to be distinctly longer in the deeper cortical layers as well as in some cells belonging to the stele. The average density of mitoses for the epidermis, the two first layers of cortex and the mother cells of central metaxylem vessel are presented in Figure 5.

Rate of Cell Formation

The rate of cell formation by an apical segment — dc/dt — (Column b, Table 1) has been obtained as a product of corresponding average values of dc/dt and dx/dt . It is obvious that this rate ought not to change beyond the cell division zone. However in this case they decrease slightly after obtaining a maximum, but the change is not significant and is due to unavoidable variation and errors of interpolation. The total rate of cell formation by a file of cells amounted to 2.3 cells per hour in epidermis, 2.17 and 2.4 cells per hour in the first and second layers of cortex respectively and 1 cell per hour in the metaxylem. The value for epidermis agrees well with the cell division rate (R_d) in a longitudinal row of cells estimated by means of Burström's (7, 8) formula $R_d = 1_r/C$ t, where $1_r/t$ — total growth rate of root, C — average length of mature cells. The average length of mature epidermal cells amounted to 270μ in our roots, which with the growth rate 0.6 mm./hr. gives cell division rate of 2.2 cells/hr. in a file.

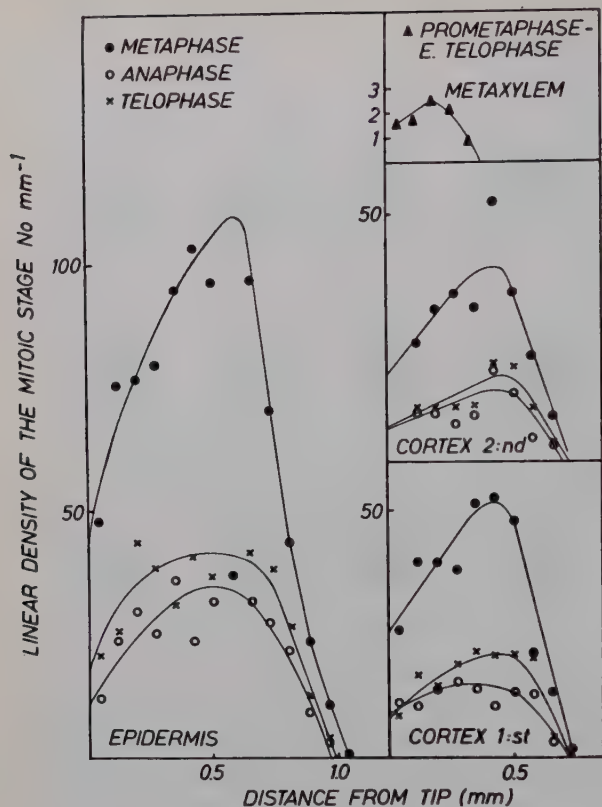


Figure 5. Density of different stages of mitosis in the investigated tissues (layers); (no. of stages in the tissue per 1 mm).

The total rate of cell formation in a given layer amounted to the following values (corrected for change of number of files along the root axis): in epidermis — 183 cells/hour, in the first layer of cortex 66 cells/hour and in the second layer of cortex 49 cells/hour.

From the dc/dt values the frequency of cell formation at x was calculated by applying the 7 point numerical derivative formula (24) to give $d(dc/dt)/dx$ at each 0.05 mm. The rate of cell formation per cell was calculated by dividing the $d(dc/dx)/dx$ values by corresponding dc/dx ones (Column c table 1).

The frequency of cell division (no. of cells \cdot mm $^{-1}$ \cdot hr $^{-1}$) changes distinctly through the cell division zone attaining a maximum at a distance of about 0.55 mm. in the epidermis and of about 0.35—0.5 mm. in the first two layers of cortex, but the cell formation rate per cell is fairly constant throughout the cell division zone. It decreases slightly only at the basal end of the cell division zone, probably because the cessation of cell divisions is not uniform (contrary to the assumption that there is the same probability of any

Table 1. Data on cell divisions in the apical meristem of wheat roots.

x mm.	dc/dt no · hr ⁻¹	$\frac{d \text{ dc/dt}}{dc}$ no/no · hr ⁻¹	Duration of mitotic stages			
			whole mito- tic cycle hr.	prometaphase and metaphase hr.	anaphase hr.	early telophase hr.
a	b	c	d	e	f	g
<i>Epidermis</i>						
0.05	0.10	0.04	25	0.46	0.13	0.18
0.1	0.21	0.04	24	0.38	0.13	0.17
0.15	0.34	0.05	21	0.34	0.12	0.15
0.2	0.48	0.05	21	0.35	0.12	0.15
0.25	0.62	0.05	22	0.37	0.13	0.17
0.3	0.75	0.05	22	0.38	0.13	0.17
0.35	0.90	0.05	22	0.39	0.13	0.17
0.4	1.05	0.05	22	0.40	0.13	0.17
0.45	1.20	0.05	22	0.41	0.14	0.17
0.5	1.34	0.05	22	0.42	0.14	0.16
0.55	1.50	0.04	22	0.43	0.13	0.16
0.6	1.65	0.04	23	0.44	0.13	0.17
0.65	1.79	0.04	26	0.48	0.14	0.18
0.7	1.92	0.04	29	0.44	0.15	0.20
0.75	2.02	0.03	33	0.39	0.15	0.20
0.8	2.13	0.03	36	0.31	0.14	0.18
0.85	2.21	0.02	43	0.29	0.12	0.16
0.9	2.28	0.02	60	0.31	0.12	0.15
<i>Cortex 1-st layer</i>						
0.05	0.12	0.04	25	0.37	0.14	0.17
0.1	0.25	0.04	24	0.38	0.14	0.17
0.15	0.40	0.05	22	0.36	0.13	0.15
0.2	0.56	0.05	21	0.35	0.12	0.15
0.25	0.73	0.05	20	0.36	0.12	0.15
0.3	0.91	0.05	21	0.38	0.12	0.16
0.35	1.09	0.05	21	0.40	0.12	0.17
0.4	1.27	0.04	22	0.42	0.12	0.17
0.45	1.45	0.04	23	0.42	0.11	0.17
0.5	1.62	0.04	23	0.39	0.11	0.17
0.55	1.80	0.04	24	0.31	0.10	0.17
0.6	1.96	0.04	28	0.27	0.09	0.17
0.65	2.10	0.02	43	0.27	0.08	0.13
<i>Cortex 2-nd layer</i>						
0.05	0.12	0.04	25	0.40	0.19	0.14
0.1	0.28	0.04	23	0.41	0.16	0.13
0.15	0.43	0.04	23	0.43	0.16	0.14
0.2	0.59	0.05	22	0.41	0.15	0.14
0.25	0.77	0.05	21	0.42	0.16	0.14
0.3	0.96	0.05	20	0.41	0.16	0.14
0.35	1.17	0.05	21	0.42	0.17	0.16
0.4	1.37	0.05	21	0.44	0.19	0.17
0.45	1.58	0.05	21	0.44	0.20	0.17
0.5	1.79	0.05	21	0.39	0.20	0.16
0.55	2.00	0.05	22	0.31	0.19	0.12
0.6	2.19	0.04	25	0.24	0.16	0.07
0.65	2.37	0.03	38	0.21	0.11	0.08
<i>Metaxylem mother cells</i>						
				from prometaphase till early telophase		
0.05	0.16	0.04	25		0.50	
0.1	0.32	0.04	25		0.61	
0.15	0.49	0.04	25		0.68	
0.2	0.65	0.04	26		0.77	
0.25	0.79	0.04	27		0.72	
0.3	0.92	0.03	37		0.66	

Table 2. *Duration of mitotic stages based on the frequency of stages on longitudinal sections.*

Tissue	Cell formation rate in the tissue no. hr ⁻¹	Number of mitosis (prometaphase-early telophase in the tissue no.	Duration of different stages (minutes)				
			from pro-metaphase till early telophase	pro-metaphase	meta-phase	ana-phase	early telophase
Epidemis	183	129	42	5	14	9	13
Cortex 1st layer	66	45	41				
Cortex 2nd layer	49	33	40				

cell of the tissue entering mitosis). The actual duration of the mitotic cycle in cells at the basal end of the cell division zone is probably considerable shorter than the estimated values would indicate.

The average time between two successive cell divisions — the duration of the whole mitotic cycle (interphase+active mitosis) — is given by the reciprocal of the rate of cell formation per cell (Column d Table 1).

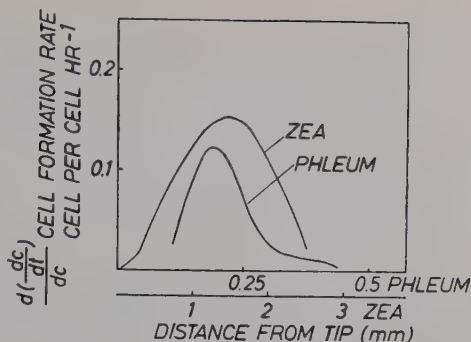
The values of linear density at different stages of mitosis obtained by means of graphical interpolation of Figure 5 were used together with the cell formation rate values for calculating the time of duration of the stages according to formula $T_m = N_m / d(dc/dt)/dx$. From these it follows that the duration of the different stages of mitosis is fairly constant with distance from the tip and there are no significant differences between the tissues. There are, of course, some fluctuations of the time values even in the same layer, especially at the basal end of the cell division zone, but they seem to be rather accidental in character.

In counting mitoses on transversal sections there is some difficulty in distinguishing between metaphase and early anaphase. It is, therefore, possible that the values for metaphase include also early anaphase. Longitudinal sections provide much better material for distinguishing between stages, but it was not possible to use them instead of the transversal ones in estimating the mitosis density. However, as soon as we know that the duration of mitosis is uniform throughout the meristem, the duration of mitosis (from prometaphase till early telophase) — T_c — may be calculated as relation of the number of mitoses to the rate of cell formation in the tissue, while the duration of different stages may be estimated as fractions of T_c proportional to the relative number of cells in the given stages found on longitudinal sections.

The proportion of different stages on longitudinal sections are: prometaphase 13 : metaphase 33 : anaphase 23 : early telophase 31 (on basis of 2.775 counted mitoses).

The results of the calculations are presented in Table 2. It can be seen that the duration of different stages agree well with the values in Table 1.

Figure 6. Rate of cell formation per cell plotted against distance from tip for Phleum — according to Goodwin and Avers (16). — and for Zea — according to Erickson and Sax (13).



Discussion

There are no data in the literature which are directly concerned with the duration of the over-all mitotic cycle at different distances from tip. However, it is possible to calculate this duration easily as a reciprocal of the rate of cell formation per cell from the papers of Erickson *et al.* (12, 13), Hejnowicz (19) and Goodwin *et al.* (16). These authors reached the conclusion that this rate is not constant in the cell division zone (Figure 6). Thus the time of mitotic cycle would not be constant.

The papers of Erickson *et al.* as well as the author's preceeding one contain all data necessary for calculating the duration of the different stages of the mitotic cycle according to the method presented above. From these works it would appear that the duration of the separate stages of mitosis would be different along the root axis. This is in disagreement with the present results.

This discrepancy probably is not due to the fact that different species are studied but rather to the accuracy in determining the growth rate in the cell division zone. In the earlier papers (13, 19) it was based to a larger extent on extrapolation from the data on the region further away from the tip than on original data for this zone. This is stressed in the author's paper on the growth of Phleum (19), but is also evident from data of Erickson *et al.* (12, 14). As a matter of fact, there are some difficulties in (accurately) estimating the growth in the apical part of the growing zone, because elongation in this part is very slow; and such a method of recording growth as the ones used by Erickson and by the author in the investigation of Phleum root are not adequate for this region.

Goodwin and Avers (16) have obtained data on cell division rates from direct observations of the epidermis of living roots. In this case the discrepancy may be due to the fact that data on cell divisions were not numerous enough for computations.

There is a rather great dispersion of individual data in the apical part of the roots in the present investigation as is shown in Figure 2. This is due to the small displacement of the points in this part as compared with the accuracy of measuring. However, there is little doubt that the displacement is proportional to the distance from the tip, which means that the relative growth rate is constant in the apical region.

The constancy of growth rate in the cell division zone was found for the first time by Brumfield (5) for Phleum root (5.8 % per hour). As the analysis of Brumfield's method (Hejnowicz 19) has shown that it is fully correct for the apical part of the meristem, the statement is quite reliable. The constancy of growth in the cell division zone in both Triticum and Phleum roots suggests that this is probably a general feature.

There is one problem which must be taken into account in connection with the growth rate in the cell division zone. Cloves (10, 11) in an excellent investigation with labelled phosphorus, sulphur and isoleucine has demonstrated that the rate of DNA synthesis as well as the protein synthesis and similarly the rate of cell division are much lower in a group of cells forming the so-called initial centre than in other cells of the division zone. The same result was obtained by Rubideau and Mericle (25) in respect to the carbohydrate metabolism with C_{14} given as CO_2 in light and by the present author with metabolism of labelled sulphur in wheat roots (unpubl. data). This means that the growth in the initial region is lower than in the other part of cell division zone. The existence of this gradient of growth rate inside the cell division zone also follows from the study of the relation between the pattern of growth and the pattern of cell arrangement (Hejnowicz 18). This gradient, however, may be limited to a very short distance from the tip so that the inactive centre found by Cloves would be quite sufficient for explaining the cell arrangement in the root meristem of Triticum as well as of Angiosperms in general. The statement of constancy of growth rate in the cell division zone excludes the small group of cells located just at the tip.

Returning to the duration of mitosis it must be stressed that the present results indicating uniform duration throughout the root meristem are not all surprising. This has been presumed by many botanists, who used the distribution of mitoses as a measure of cell division rate in different parts of the meristem (1, 9, 21, 27) or calculated the duration of mitoses in root meristems (2, 3, 4, 17, 22). The present results have proved this assumption experimentally.

The values for the duration of the different phases given in the present paper may be compared with data of the literature. It is well known that the duration of mitosis varies in different tissues (known for animals) and between organisms. It also varies with temperature and chemical treatments.

Thus it is necessary to limit the comparison to the duration of mitoses in root meristems. The first evaluation of mitosis duration in root has been made by Laughlin (22) for root tips of *Allium cepa* grown at different temperatures. One may question the reliability of his absolute values, but it follows clearly from his data that the anaphase takes about twice as long as the metaphase in contrast to *Triticum* roots. This difference, however, is not surprising as the ratio of duration of metaphase to anaphase seems to differ between organisms (Sharp 26).

Brown (4) has estimated the duration of the mitotic cycle in pea roots growing at different temperatures using maceration technique for estimating the rate of cell formation and mitotic index. His data for 15°C and 20°C may be compared with those given here, and it can be seen that they do not differ significantly save for the duration of the anaphase which is distinctly shorter in wheat than in peas.

Summary

Data on the growth of the apical part of wheat seedling roots have been obtained by means of photographic recording of the root surface. From these it follows that the growth rate is constant throughout the cell division zone.

Rates of cell division in the root meristem of wheat have been determined by means of the Goodwin-Erickson method. The absolute values are determined for epidermis, the first two layers of cortex (separately) and central metaxylem mother cells.

The meristematic activity measured as rate of cell formation per cell is fairly constant throughout the meristem (excluding the inactive centre near the tip).

A method is developed for determining the duration of the mitotic cycle stages in different part of the meristem. The duration of the stages is constant throughout the meristem. It proves the assumption that the distribution of mitoses is a measure of the frequency of cell division in different parts of the meristem.

The author is much indebted to Prof. H. Burström for valuable discussions and support during the course of this work. The investigation has been supported by a grant from the Swedish Science Research Council.

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Das Permeationsvermögen des Pentaerythrits verglichen mit dem des Erythrits

Von

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(Eingegangen am 24. Oktober 1958)

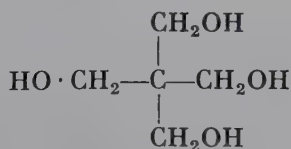
1. Einleitung

Der eventuelle Einfluss der *Form* der permeierenden Moleküle auf ihr Permeationsvermögen ist bisher sehr wenig diskutiert worden (vgl. Wartiovaara und Collander 1959). Experimentelle Erfahrungen, die geeignet wären diese Frage zu beleuchten, gibt es anscheinend auch nur sehr spärlich. Doch scheint es, wie wenn Verbindungen mit reich verzweigten Molekülen — z.B. tert. Butylalkohol (=Trimethylcarbinol), Triacetin sowie Trimethyl- und Triäthylcitrat — etwas langsamer durch Nitella-Protoplasten permeierten als unverzweigt kettenförmige Moleküle mit ähnlicher Lipoidlöslichkeit und gleichem Molekulargewicht (Collander 1954). Auffallend ist auch die hiermit analoge Beobachtung, dass Pentaerythrit langsamer als Erythrit in die Protoplasten eines Photobacteriums eindringt (Collander 1956), trotzdem die Lipoidlöslichkeit des Pentaerythrits ein wenig grösser als die des Erythrits ist.

Es lag nun nahe, Erfahrungen über das Verhalten gerade des zuletzt genannten Substanzaaaes andersartigen Protoplasten gegenüber zu sammeln. Denn chemisch und physikochemisch gleichen sich ja diese Verbindungen weitgehend, wogegen sie hinsichtlich der Form ihrer Moleküle stark voneinander abweichen, wie bereits ein Blick auf die betreffenden Strukturformeln zeigt:



Erythrit



Pentaerythrit

Physiol. Plant., 12, 1959

Tabelle 1. Verschiebung (Δ_{Erythrit} bzw. $\Delta_{\text{Pentaerythrit}}$) der plasmolytischen Grenzkonzentration der Rhoeo-Zellen in Erythrit- und Pentaerythritlösungen. Alle Konzentrationen in mol/l.

Datum	Versuchsdauer (Stunden)	Δ_{Erythrit}	$\Delta_{\text{Pentaerythrit}}$	$\Delta_{\text{Erythrit}}/\Delta_{\text{Pentaerythrit}}$
19. 6.54	45	0.07	0.025	2.8
21. 9.54	41	0.025	0.00	∞
21.11.54	43	0.035	0.015	2.3
23.11.54	39	0.03	0.01	3.0
28. 3.57	48	0.04	0.008	5.0
29. 3.57	50	0.035	0.015	2.3
18. 5.57	45	0.055	0.025	2.2
11. 9.58	48	0.065	0.003	22
14. 9.58	43	0.10	0.01	10

2. Versuchsobjekte und Methodik

Sowohl Erythrit wie Pentaerythrit permeieren im allgemeinen nur sehr langsam durch lebende Protoplasten. Aus diesem Grunde schien es nicht zweckmässig, ihr Verhalten etwa grossen Characeen-Zellen gegenüber zu untersuchen. Vielmehr wurde das Eindringen dieser Verbindungen in mikroskopisch kleine Zellen auf grenzplasmolytischem Wege verfolgt. Es wurden also Zellen möglichst gleichmässigen osmotischen Wertes in Erythrit- und Pentaerythritlösungen abgestufter Konzentration gebracht und der allmähliche Anstieg der plasmolytischen Grenzkonzentration während etwa 24—48 Stunden verfolgt.

Drei Versuchsobjekte kamen zur Anwendung, und zwar 1. Epidermiszellen der Blattmittelrippe von *Rhoeo discolor*, 2. Blattzellen von *Elodea densa* sowie 3. Mesophyllzellen von *Vallisneria spiralis*.

In den Versuchen mit *Rhoeo* wurde in allem wesentlichen die Methodik von Bär-lund (1929) befolgt.

Für die Versuche mit *Elodea* wurden Blätter mittleren Alters benutzt. Kurz vor dem Versuchsbeginn wurden sie mit einer Rasierklinge mitten quer durchgeschnitten. Nur das Verhalten von etwa 6 der Schnittfläche am nächsten gelegenen Zellreihen — selbstverständlich mit Ausnahme der durch den Schnitt beschädigten — wurde beachtet.

Der mittlere Teil der *Vallisneria*-Blätter wurde zuerst in etwa 5 mm lange Stücke zerlegt und diese dann zwischen Daumen und Zeigefinger durch einen oberflächenparallelen Schnitt halbiert. Für die Versuche wurde jedesmal die dickere Hälfte genommen und mittels der Wasserstrahlpumpe teilweise entlüftet. (Vgl. Hurch 1933.)

Die plasmolisierenden Lösungen wurden teils in glasdestilliertem Wasser, teils in durch Aufkochen entchlortem Leitungswasser hergestellt. Da Pentaerythrit etwas schwerlöslich ist, wurden Erythrit und Pentaerythrit nicht in höheren Konzentrationen als 0,4 mol/l benutzt. Wenn höhere osmotische Werte nötig waren, wurde die Partialdruckmethode benutzt, und zwar unter Benutzung von Mannit als Zusatz. In den Versuchen mit *Rhoeo* waren die Lösungen im allgemeinen um 0,01 mol/l, in denen mit *Elodea* und *Vallisneria* dagegen um 0,02 mol/l abgestuft.

Die Temperatur im Versuchsraum schwankte in den einzelnen Versuchen zwischen etwa 18 und 22° C. Da aber die Versuche mit Erythrit und Pentaerythrit immer

Tabelle 2. Verschiebung der plasmolytischen Grenzkonzentration der *Elodea*-Zellen in Erythrit- und Pentaerythritlösungen. Alle Konzentrationen in mol/l.

Datum	Versuchsdauer (Stunden)	Δ_{Erythrit}	$\Delta_{\text{Pentaerythrit}}$	$\Delta_{\text{Erythrit}}/\Delta_{\text{Pentaerythrit}}$
14. 6.54	24	0.08	0.03	2.7
17. 6.54	25	0.095	0.015	6.3
23. 6.54	28	0.15	0.03	5.0
8.11.54	24	0.045	0.005	9.0
14.11.54	42	0.073	0.013	5.6
16.11.54	43	0.045	0.015	3.0
22. 5.57	21	0.065	0.02	3.3
25. 5.57	48	0.10	0.025	4.0
3.10.58	20	0.08	0.01	8.0

gleichzeitig liefen, dürfte der Vergleich dieser zwei Substanzen doch nicht allzu sehr durch die Temperaturschwankungen beeinträchtigt worden sein.

Die benutzten Erythritpräparate stammten von E. Merck (Darmstadt), das Pentaerythrit von Eastman Kodak Co. (Rochester).

3. Ergebnisse

Die Ergebnisse der mit *Rhoeo* und *Elodea* ausgeführten Versuche sind in den Tabellen 1 und 2 zusammengestellt. Man sieht, dass die plasmolytische Grenzkonzentration bei *Rhoeo* in 48 Stunden in den Erythritlösungen meistens etwa um 0,04—0,06 mol/l, d.h. etwa um 20—30 % ihres ursprünglichen Wertes gestiegen ist. Das sind Werte, die mit den von Bärhund (1929) für dasselbe Objekt angegebenen gut übereinstimmen. Die mit *Elodea* erhaltenen Werte variieren etwas stärker, stimmen aber mit den von Marklund (1936) angegebenen einigermaßen überein. In Lösungen des weder von Bärhund noch von Marklund geprüften Pentaerythrits sind die Verschiebungen der Grenzkonzentration bei beiden Objekten bedeutend kleiner, oft sogar so klein, dass die quantitative Bestimmung der stattgefundenen Veränderung etwas schwierig war. Die in den Tabellen angegebenen Zahlenwerte für $\Delta_{\text{Pentaerythrit}}$ sowie für $\Delta_{\text{Erythrit}}/\Delta_{\text{Pentaerythrit}}$ sind daher nur als Approximationen aufzufassen. Unter allen Umständen steht aber fest, dass sich die plasmolytische Grenzkonzentration bei beiden Objekten mindestens etwa zweimal so langsam in Lösungen von Pentaerythrit als in solchen von Erythrit erhöht.

Wie aus Tab. 3 ersichtlich sind die Befunde an Mesophyllzellen von *Vallisneria* nicht ganz so eindeutig. In den ersten Versuchen schien sich nämlich die Grenzkonzentration etwa gleich langsam in Erythrit- wie in Pentaerythritlösungen zu verändern, während in den drei letzten Versuchen, die mit besonderer Umsicht ausgeführt wurden, auch bei diesem Objekt die

Tabelle 3. Verschiebung der plasmolytischen Grenzkonzentration der *Vallisneria*-Zellen in Erythrit- und Pentaerythritlösungen. Alle Konzentrationen in mol/l.

Datum	Versuchsdauer (Stunden)	Δ_{Erythrit}	$\Delta_{\text{Pentaerythrit}}$	$\Delta_{\text{Erythrit}}/\Delta_{\text{Pentaerythrit}}$
14. 6.54	24	0.11	0.09	1.2
17. 6.54	24	0.06	0.08	0.8
8.11.54	24	0.08	0.08	1.0
14.11.54	25	0.045	0.04	1.1
16.11.54	26	0.09	0.075	1.2
27. 5.57	24	0.02	0.02	1.0
21. 9.58	27	0.05	0.035	1.4
23. 9.58	23	0.035	0.02	1.6
30. 9.58	42	0.04	0.025	1.6

Erhöhung der Grenzkonzentration deutlich langsamer in Pentaerythrit- als in Erythritlösungen erfolgte. Ob die Diskrepanz zwischen den ersten und den letzten Versuchsergebnissen auf etwaigen Beobachtungsfehlern beruht oder ob sie tatsächliche Unterschiede im Verhalten des Versuchsobjekts widerspiegelt, kann nicht sicher entschieden werden, denn es ist nicht leicht ganz genau festzustellen, wann sich die letzten Spuren der Plasmolyse in den *Vallisneria*-Schnitten ausgeglichen hatten. Dass aber wenigstens in den drei letzten Versuchen die Plasmolyse langsamer in Lösungen des Pentaerythrits als in denen des Erythrits zurückging, dürfte kaum zu bezweifeln sein.

4. Besprechung

Vorausgesetzt, dass die zeitliche Erhöhung der Grenzkonzentration als ein Mass der Permeationsgeschwindigkeit der betreffenden Substanz gelten kann, würde man aus den im vorigen Abschnitt dargestellten experimentellen Ergebnissen zu schliessen haben, dass Pentaerythrit mindestens etwa zweimal langsamer als Erythrit durch die untersuchten Protoplasten von *Rhoeo* und *Elodea* permeiert, während im Falle der *Vallisneria*-Zellen der Unterschied zwar gleichsinnig, jedoch etwas kleiner sein dürfte.

Wie gross ist nun aber die Wahrscheinlichkeit, dass jene Voraussetzung tatsächlich erfüllt ist?

Bekanntlich hat Bogen (vgl. Bogen und Follmann 1955, Bogen 1956) die Meinung vertreten, dass man mittels plasmolytischer Methoden nur einen *scheinbaren* osmotischen Wert des Zellinhaltes bestimmt und dass wir „bisher noch keine Methode besitzen, die es uns erlaubt, die osmotische Aufnahme der Anelektrolyte allein und exakt zu messen“. Wenn auch diese Behauptungen weit über das Ziel schiessen dürften (vgl. Höfler und Url 1958, War-

tiovaara und Collander 1959), ist doch einzuräumen, dass es gerade im Falle solcher extrem langsam permeierenden Stoffe wie Erythrit und Pentaerythrit fast unmöglich sein dürfte einwandfrei zu beweisen, dass sie allein oder auch nur hauptsächlich auf rein osmotischem Wege in die Zellen aufgenommen werden und dass die in Lösungen solcher Stoffe nur sehr langsam stattfindende Erhöhung der plasmolytischen Grenzkonzentration von dem allmählichen Eindringen des Plasmolytikums verursacht ist. Immerhin lässt sich wohl auf Grund der hier dargestellten Versuchsergebnisse sagen, dass Pentaerythrit *wahrscheinlich* bedeutend langsamer als Erythrit in die untersuchten Zellen — und zwar besonders in die von Rhoeo und Elodea — eindringt.

Andererseits beträgt nach Collander (1950) der Verteilungskoeffizient Äthyläther/Wasser für Pentaerythrit 0,00030, für Erythrit dagegen nur 0,00011, während die Verteilungskoeffizienten im System *iso*-Butanol/Wasser für Pentaerythrit 0,14 und für Erythrit 0,037 sind. Von den beiden in Rede stehenden Substanzen scheint also Pentaerythrit ein wenig mehr lipophil (=weniger hydrophil) zu sein.

Hier liegt somit ein neues Beispiel dafür vor, dass sperrig gebaute Moleküle langsamer permeieren als kettenförmige Moleküle entsprechender Lipidlöslichkeit und ähnlicher Molekülgrösse.

Hinsichtlich der theoretischen Bedeutung dieses Befundes sei auf die Ausführungen bei Wartiovaara und Collander (1959) hingewiesen.

Summary

Pentaerythritol is known to possess a slightly greater lipid solubility than erythritol. In spite of this, epidermal cells of Rhoeo and leaf cells of Elodea were found to deplasmolyze considerably more slowly in solutions of pentaerythritol than in solutions of erythritol. (Observations on mesophyll cells of Vallisneria were somewhat more ambiguous but pointed in the same direction.)

These findings seem to corroborate the view that richly branched molecules have a smaller permeation power than less bulky molecules of equal lipid solubility and equal molecular volume.

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The Possible Significance of Pectic Enzymes in Root Hair Infection by Nodule Bacteria

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The mechanism by which the nodule bacteria invade the root hairs of susceptible leguminous plants has remained rather obscure up to the present time despite several efforts to demonstrate enzymes active in this process. As early as in 1888, Beijerinck concluded that the cell wall substance was not attacked, as his attempts to grow nodule bacteria on a cellulose substrate were unsuccessful. The matter was re-examined by McCoy (1932) who studied the ability of 17 different cultures of rhizobia to grow on media containing cellulose, pectin, or calcium pectate. In no case was any growth at the expense of these substances observed. Smith (1958) also reports negative results with four strains on a pectin medium.

However, the above-mentioned results can not be considered entirely conclusive. First, the inability of the nodule bacteria to grow on cellulose or pectin as sole sources of carbon does not exclude the possibility that they can under certain conditions effect a depolymerization or some other change of these substances. Such a phenomenon has been shown to occur in cultures of cellulose-decomposing bacteria which are able to hydrolyze but not to metabolize xylan (Sørensen 1957). Second, the enzymes in question might be formed by the bacteria only in the presence of growing roots, as a specific adaptive response. Finally, as suggested earlier (Fåhraeus 1957), the nodule bacteria might well be unable to synthesize any cell-wall dissolving enzymes but might induce the production of such enzymes by the host plant. This could be effected by secreting indole-3-acetic acid (IAA) or some other auxin. IAA is in fact formed by nodule bacteria according to Chen (1938),

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Georgi and Beguin (1939), and others, and recent work (Neely *et al.* 1950, Bryan and Newcomb 1954, Glasziou 1957) suggests a specific interaction between plant hormones and pectic enzymes.

In any case, infection seems to be intimately associated with the growth process of the host plant (Nutman 1956). Ekdahl (1953), who studied in detail the growth of root hairs (in wheat), concluded that pectic enzymes of the plant play a decisive role for the growth of the hairs.

The above considerations made a re-examination of the role of pectic enzymes in infection desirable. In our studies, we used seedlings of leguminous plants inoculated with nodule bacteria. The seeds were allowed to germinate in water, and this liquid, as well as plant extracts, were examined for presence of pectic enzymes. Pectin methyl esterase (PME) was determined by titration. For the measurement of polygalacturonase activity (PG), the viscometric technique was employed. No attempt has been made to differentiate between polygalacturonase and pectin depolymerase according to Wood (1955); polygalacturonase is used here in the sense of Deuel and Stutz (1958).

Materials and Methods

Seeds of the following plants were used: white clover (*Trifolium repens*) variety Morsö, hybrid clover (*T. hybridum*) variety Svea, lucerne (*Medicago sativa*) variety Tuna, and yellow pea (*Pisum sativum*) var. Torsdags II, all from Svalöf, Sweden. The seeds were disinfected in erlenmeyer flasks by successive treatment with 95 % ethyl alcohol and equal parts of 0.2 % formaldehyde and 0.2 % HgCl_2 and were then washed in several changes of sterile distilled water. They were transferred to 10 cm. petri dishes and were allowed to germinate at room temperature (20°C) in a shallow layer of distilled water (ca. 5 ml.). Each dish received a quantity of about 200—300 seeds of clover, or 150—200 seeds of lucerne, respectively.

After 2 days, the seedlings were inoculated with suspensions of *Rhizobium* cells. The cultures used were pure and effective strains of *Rh. trifolii* (nos 211, 220 and 226), *Rh. meliloti* (nos 4 and 27), *Rh. leguminosarum* (no. 311), *Rh. lupini* (mixture of nos 623 and 624) and *Rh. phaseoli* (mixture of nos 453 and 458). Some dishes were always left uninoculated as controls. The dishes were placed at 25°C. under a fluorescent lamp.

After an additional 2 days aliquots were taken out for measurement of the enzymatic activity. In addition to uninoculated controls, also boiled samples were tested for activity. Before testing, the liquid was filtered through a layer of cotton. In some experiments the solutions were passed through bacterial filters, but in no case was any activity recovered from such cell-free filtrates. In most experiments, the seedlings were also shaken for one hour with 0.1 M phosphate buffer containing 10 % NaCl (pH 7.8) to extract pectin-decomposing enzymes (Glasziou 1957). This solution was dialyzed against distilled water before testing.

Extreme care was always exercised to avoid contamination by foreign bacteria and moulds. The purity was ascertained by regular microscopic examination of the

test solutions and roots, and frequent transferences to plain agar media were made to detect possible contaminants. Infected cultures were always discarded.

For polygalacturonase assay, the drop in viscosity of a pectin solution was followed in an Ostwald viscometer at 30°C. The test solutions had the following composition: 2 % pectin solution (freshly prepared for each experiment) 5 ml., 0.1 M acetate buffer pH 5.0 2 ml., 5 % NaCl 1 ml., enzyme-containing solution 3 ml. In most experiments toluene was added as an antiseptic. The pectin solution was mixed with buffer and NaCl and allowed to equilibrate in the constant temperature bath. The enzyme solution was kept separately and adjusted to the same temperature. At zero time, the two solutions were mixed and immediately filled into the viscometer. Measurements were ordinarily run for 1 hour, sometimes also for 24 hours.

The results have been expressed as percentage decrease in viscosity according to the formula (Landis and Redfern 1947):

$$P = \frac{t_0 - t_{60}}{t_0 - t_f} \times 100, \text{ where}$$

P = % decrease in viscosity

t_0 = initial flow time

t_{60} = flow time after 60 min.

t_f = calculated flow time of completely hydrolyzed pectin.

Two different pectin preparations have been used, in the first experiments a citrus pectin from Eastman Kodak (no. P 2569), later usually low methoxyl citrus pectin from Coleman & Bell (no. 7366). The latter preparation showed a flow time under the above test conditions of ca. 200 sec. (water ca. 40 sec.).

Pectin methyl esterase activity was measured by titration of the test solutions with 0.005 N NaOH to pH 7.5 (Radiometer pH-meter with glass electrode). The reaction mixtures had the following composition: 1 % pectin solution 25 ml., 5 % NaCl 2.5 ml., enzyme solution 4 ml., pH adjusted to 7.5 by addition of 0.1 N NaOH. Measurements run for 1 hr.

In the PME assays, the pectin preparation Ea P 2569 was used throughout.

Experiments and Results

1. *Polygalacturonase activity*

Seedlings of leguminous plants were combined with different types of nodule bacteria and samples taken out were tested for PG activity. Four plant species (white clover, hybrid clover, lucerne, and pea) were first cultured in the presence of their specific bacterial symbionts (Table 1). In a second experiment, clover seedlings were combined with clover bacteria as well as with bacteria of other cross-infection groups (Table 2). Lucerne seedlings were similarly grown together with strains of lucerne and clover bacteria (Table 3).

It is evident from Tables 1—3 that a definite PG activity regularly occurs

Table 1. *Formation of PG in associations of some leguminous plants and their specific nodule bacteria.* PG activity expressed as % decrease in viscosity of a 1 % pectin solution after 60 min.

Plant species	Bacterial strain	PG activity in liquid
<i>Trifolium repens</i>	uninoculated	— 1.1 ¹
" "	clover 220	13.1
" <i>hybridum</i> ..	uninoculated	— 1.1
" " ..	clover 220	11.1
<i>Medicago sativa</i>	uninoculated	6.4
" "	lucerne 4	5.0
<i>Pisum sativum</i>	uninoculated	0.7
" "	pea 311	16.4

¹ Negative values: increase in viscosity

in associations of seedlings and the appropriate bacterial strains, although somewhat variable results have been obtained. Very little or no activity was found in the uninoculated controls and also in the associations of plants and non-infective bacteria. These experiments have been repeated several times, and consistent results have been obtained. From Tables 2—4 it is apparent that an extraction of the plant material with phosphate buffer gives a large additional activity especially in clover experiments; thus a considerable part of the enzyme quantity seems to be adsorbed by the plants.

It is well-known that an addition of nitrate stops the infection of legumes by rhizobia. In order to determine if there was a similar effect on the production of polyglacturonase, experiments were made with nitrate added to clover and lucerne seedlings in association with their specific bacteria (Table 4). From this Table it is seen that, in the clover experiment, the formation of PG is totally suppressed at a concentration of 2 mg. $\text{NaNO}_3/\text{ml.}$, and to a

Table 2. *Formation of PG in associations of white clover and different nodule bacteria.* PG activity expressed as in Table 1, but measured after 24 hrs. Both liquid and plant extract analyzed. Blanks (=boiled enzyme) subtracted.

Bacterial strain	PG activity in	
	liquid	extract
Uninoculated	0.8	0.2
Clover strain 211	6.9	10.3
" " 220	7.3	17.6
" " 226	11.6	15.8
Pea " 311	3.4	1.9
Lucerne " 4	0.1	0.8
Lupine " 623+624 ..	— 1.6	2.0
Bean " 453+458 ..	— 0.2	4.1

Table 3. *Formation of PG in associations of lucerne seedlings with lucerne and clover bacteria.* PG activity expressed as in Table 1. Viscosity measurements after 1 hr and 24 hrs. Blanks subtracted.

Bacterial strain	Liquid		Extract	
	1 hr	24 hrs	1 hr	24 hrs
Uninoculated	0.6	3.1	1.3	4.5
Clover strain 211	— 0.8	— 0.1	0.6	— 0.2
" " 220	1.0	0.4	3.6	6.2
" " 226	4.2	3.3	— 0.4	5.7
Lucerne strain 4	10.4	22.1	6.4	25.6
" " 27	7.2	14.6	0.9	30.1

Table 4. *Influence of nitrate on the formation of PG in associations of white clover and lucerne with their specific bacteria* (clover strain 220, lucerne strain 27). PG activity expressed as in preceding Tables. Blanks subtracted.

Treatment	Clover experiment				Lucerne experiment			
	Liquid		Extract		Liquid		Extract	
	1 hr	24 hrs	1 hr	24 hrs	1 hr	24 hrs	1 hr	24 hrs
Uninoculated, without nitrate	1.2	— 1.3	0.0	1.2	2.6	3.2	2.5	0.2
" with 2 mg NaNO ₃ /ml	0.4	0.4	1.6	3.7	3.5	3.1	1.9	11.0
Inoculated, without nitrate	6.9	13.1	15.2	21.3	14.3	44.6	8.8	13.8
" with 0.2 mg NaNO ₃ /ml	— 0.1	6.8	0.6	— 1.6	18.3	29.6	7.1	13.1
" with 2.0 mg "	— 2.1	— 1.3	0.1	— 11.5 ¹	6.6	7.8	3.8	7.0

¹ This high negative value is probably due to some experimental error.

Table 5. *Formation of PME in associations of white clover with different nodule bacteria.* PME activity expressed as ml 0.005 N NaOH after 60 min.

Bacterial strain	PME activity		
	liquid	extract	sum
Uninoculated	3.65	1.40	5.05
Clover strain 211	2.20	2.80	5.00
" " 220	2.16	3.10	5.25
" " 226	2.20	2.85	5.05
Pea strain 311	2.25	2.15	4.40
Lupine strains 623+624	1.95	2.40	4.45
Lucerne strain 4	2.10	2.25	4.50
Bean strains 453+458	1.80	2.65	4.45
Clover strain 211 (boiled)	0.20	0.20	0.40

Table 6. *Formation of PME in associations of lucerne with clover and lucerne bacteria. PME activity as in Table 5.*

Bacterial strain	PME activity		
	liquid	extract	sum
Uninoculated	3.15	3.90	7.05
Lucerne strain 4	2.25	4.65	6.90
" " 27	2.00	4.65	6.65
Clover strain 211	2.45	3.75	6.20
" " 226	2.65	3.85	6.50
Lucerne strain 4 (boiled)	0.25	0.25	0.50

large extent even at 0.2 mg./ml. In the lucerne experiments the inhibition was less pronounced although at the higher nitrogen concentration still very strong.

Preliminary experiments performed in order to establish the optimum pH range for PG formation gave rather small differences in the range 4.5—7.0 with a peak at pH 5.0. Subsequent work has been conducted at this pH.

2. *Pectin methyl esterase activity*

Tables 5—7 show the PME activities obtained in experiments arranged in a similar manner as those described above. Tables 5—6 illustrate experiments with clover and lucerne seedlings, respectively. Contrary to the results with PG, PME is found in about the same amounts in both inoculated and uninoculated samples. There is, however, a difference in the distribution of the enzyme, a larger part being adsorbed to the plants in samples inoculated with the appropriate bacteria.

Table 7 illustrates an experiment made in order to study if indole acetic acid exerted some influence on the binding of PME, in analogy with the findings of Glasziou (1957). In fact, a certain effect of a 10^{-10} M solution of IAA was noticed which is comparable to the effect of the clover strain 220. On the other hand, IAA was without influence on the PG activity.

Table 7. *Formation of PME and PG in white clover inoculated with clover bacteria or in the presence of 10^{-10} M indole acetic acid. PME and PG activities expressed as in preceding Tables.*

Treatment	PME activity 1 hr			PG activity, 24 hrs liquid
	liquid	extract	sum	
Uninoculated	3.15	3.25	6.50	2.5
+clover strain 220	2.25	4.05	6.30	26.2
+IAA	2.05	4.00	6.05	1.0
+clover strain 220 (boiled) ..	0.20	0.20	0.40	1.0

Discussion

Evidence has been presented to show that pectic enzymes are formed in associations of legume seedlings and nodule bacteria. Polygalacturonase (PG) was found in significant amounts only when seedlings were grown in combination with their appropriate bacteria, whereas pectin methyl esterase (PME) occurred in all associations irrespective of the type of nodule bacteria present, and also in aseptically grown control plants.

The observed correlation between infectiveness of the bacteria and occurrence of PG in the associations seems to indicate a special function of PG in the infection process. It is well known that infection normally takes place at the tip of young growing root hairs. Recent work (Ek Dahl 1953) attributes to the pectic enzymes an important role in the growth of root hairs, the enzymatic activity resulting in "a softening of the apical wall". Apparently a weakening of the cell wall is also a necessary prelude to infection by nodule bacteria. Nutman (1956) has launched the hypothesis that the bacteria participate in root hair growth by "building themselves into and through the primary wall" or by "inducing a reorientated growth of the host cell wall to give an infection thread by invagination". Even so, an increased PG activity might be necessary to initiate the changed growth process.

On the basis of our experiments, it is not possible to decide if the PG is formed by the bacteria or by the plants as a response to bacterial action. One fact may be taken as an indication that the plants, rather than the bacteria, synthesize the PG. It was shown in Table 4 that nitrate inhibits the formation of PG, and it would be difficult to explain such an effect of nitrogen on the formation of a bacterial enzyme. On the other hand, nitrate is known to stop the infection of legumes by otherwise infective strains. It also inhibits the curling of root hairs which precedes infection (Thornton 1936). The explanation might be an increased resistance of the host plant which is due to a more favourable C:N ratio in the plant (Thornton 1936). The barrier preventing infection as well as PG formation is certainly of plasmatic origin.

By assuming that the polygalacturonase is formed by the plants it is also easier to understand why the nodule bacteria fail to grow on substrates containing pectin as the sole source of carbon.

The bacteria might be thought to secrete a substance which penetrates the root hair wall and induces the formation of PG (or more PG) by the plant. In the introduction (p. 145), indole acetic acid was mentioned as a possibility; there is evidence that this compound is produced by nodule bacteria and that it may influence the occurrence of pectic enzymes. However, many types of nodule bacteria and also related species like *Agrobacterium radiobacter* form

IAA (Georgi and Beguin 1939), and this compound would therefore not cause such a specific response as has been noted in our experiments. It would seem more plausible that a compound with higher specificity is acting, for instance some kind of polysaccharide. Some arguments in favour of this idea may be put forward. The rhizobia form abundant slime of a polyuronide nature which is chemically related to the *Pneumococcus* polysaccharides (Hopkins, Peterson and Fred 1930, Bray, Schlüchterer and Stacey 1944). Among these polysaccharides, there exists a great variation in antigenic properties and chemical structure (Heidelberger 1956). Cross reaction between a *Rhizobium* polysaccharide and a type III anti-*Pneumococcus* serum has been reported (Lawson and Stacey 1954). It is interesting to note that galacturonic acid units occur in at least one of the *Pneumococcus* polysaccharides. A structural similarity between the *Rhizobium* polysaccharide and the plant pectin might well be of particular importance as suggested by Nutman (1956).

If a certain differentiation of polysaccharide structure occurs within the genus *Rhizobium* as in *Pneumococcus* it could possibly account for the specificity in infection properties and induction of PG formation. Dubos (1939) reports a relevant case of enzyme induction by a specific *Pneumococcus* polysaccharide (type III). In his experiments, enzyme formation was induced by the specific polysaccharide alone and not by the very similar type VIII polysaccharide which, nevertheless, exhibited serological cross reaction with type III.

As a first step in the infection of root hairs by rhizobia, the bacterial polysaccharide might combine with a specific cell component of the plant to form an "organizer" controlling the synthesis of PG. By an increased PG production, a partial depolymerization of the cell wall pectin would take place thus facilitating the entry of the bacteria. On the other hand, a polysaccharide different in some essential point will not combine with the specific cell component, and no PG (or less PG) will be formed.

Regarding the formation of PME, only small differences in the total amount of enzyme were found between aseptically grown plants and inoculated plants. More PME was, however, bound to the plant material in the inoculated series. This fact can perhaps be interpreted as an IAA effect of bacterial origin and is then in accordance with the results obtained by Glasziou (1957). An experiment with IAA (Tab. 7) gave results consistent with this assumption, but more data are required for the evaluation of this finding.

On the basis of our results, no particular role in the infection process can be attributed to PME. This enzyme might be responsible for the deformation of root hairs caused by bacterial secretions, since this effect

is not restricted to infective bacteria (McCoy 1932). This idea is, however, difficult to reconcile with the fact that nitrate completely stops the deformation (Thornton 1936). According to our preliminary results (unpublished), nitrate has no effect on the proportion bound to free PME in associations of lucerne seedlings with their specific bacteria.

Summary

The occurrence of pectic enzymes in associations of legume seedlings and nodule bacteria was investigated. Polygalacturonase activity (PG) was determined viscometrically and pectin methyl esterase activity (PME) by acidimetric titration. The main results may be summarized as follows.

1) PG in varying amounts was regularly found in associations of seedlings and the appropriate bacteria (*e. g.*, clover plants with clover bacteria);

2) PG was not found in significant amounts if non-infective bacteria were used (*e. g.*, clover with lucerne bacteria, or vice versa). Nor was it found in uninoculated control plants;

3) PME was found both in inoculated and uninoculated series. However, the presence of nodule bacteria effected a stronger binding of PME to the plant material.

The significance of these findings is discussed. It is concluded that polygalacturonase takes part in the infection process. PME seems to be produced by the plant only; concerning PG, it cannot be definitely stated if it is synthesized by the bacteria or by the plant as a response to bacterial action, although the latter appears more probable.

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Tryptophane Contents of New Potato Tubers Forced by Rindite in the Different Phases of the Germination

(Physiological studies of the potato XIV)

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Introduction

Tryptophane (TTP) can be found in the potato tuber only in a small quantity (0.04 %) as it has been already stated by Roth (1939) and de Man and de Heus (1948). Few papers so far deal with the TTP contents of the potato tuber, although this amino acid indirectly plays an important role in the sprouting of the tubers and in sprouting processes in general. Irion and Fischnich (1952) examined the effect on the potato tubers of different concentrations of rindite, a mixture of ethylene chlorhydrine, ethylene dichloride and carbon tetrachloride (7 : 3 : 1), which very rapidly breaks the dormancy of the new potatoes (Denny 1945). They found that, of the amino acids the TTP content increases partly with the concentration of rindite and partly with the progress of sproutings. Studying the amino acid contents of healthy and virus infected tubers Schuphan (1950) stated that considerable changes may be observed in the quality of the amino acids due to virus infection, *i.e.* the appearance of TTP, lysine, arginine and histidine in large quantities is particularly striking. Avery and Berger (1943), Wildmann, Ferri and Bonner (1947), Bonner and Wildmann (1947) and others demonstrated that TTP is the precursor of the growth substance β -indolyl acetic acid (IAA). TTP turns through indolylpyruvic acid (IPA) or tryptamin (TNH_2) into β -indolyl acetaldehyde (IAAld) and this into β -indolyl acetic acid (IAA).

Consequently the TTP is a precursor wherefrom active growth substances arise due to reductive and oxidative transformations.

By means of paper chromatographic examinations of the free amino acid contents of the potato juice the author (Szalai 1957 b and 1959 a) found increasing intensities of the tryptophane-valin-amino butyric acid spots during the sprouting. The fact that TTP, as an auxin precursor, plays an important role in the sprouting of the potato tubers, justified a more thorough examination of the quantitative changes of the TTP.

The study has aimed at a determination of the relation between free and bound TTP in the different parts of the potato tubers during the sprouting, and of whether quantitative changes can have some bearing on that of the auxins.

Method

The potato, variety "rose of Kiszvárd" was used for the experiments. The artificial sprouting of the new tubers was forced with 0.8 ml./kg. rindite, 48-hour treatment. The tubers, after treatment, were planted with the controls in wet sand at 20—21°C. The free and bound TTP contents of the tubers were determined (Roth's method 1939) on 1, 8, 15, 22, and 29, days following treatment. The tubers of the controls were kept under similar conditions, analysed, however, only on the 1 and 29 day.

Experimental Results

In sectors of the tubers compared with that of the controls significant TTP-changes could be observed due to the rindite treatment (Figure 1). The total TTP content in the sectors "A", "B" and "C" (Figure 1) is rapidly increasing at the beginning of the sprouting, then decreasing from the 22th day. The quantity measured in the sector "D" on the 8th day is not in keeping with the standard. The extremely low value measured on the 29th day is very striking.

The relation between the free and bound TTP is as follows. In the sector "A" the quantity of the free TTP in 100 g. fresh weight is 5.4, 7.2, 14.8., 23.6, and 0.3 mg. Higher values were obtained in the sector "B" (6.4, 9.2, 16.8, 26.4, 0.6 mg.). On the other hand, the free TTP was less in the sectors "C" and "D" and during the sprouting it increased to a considerably smaller degree. In the sector "C" it was maximally 14.8 mg., in "D", however, only 10.6 mg. While the quantity of the free TTP was all the time increasing except on the 29th day, that of the bound TTP began to decrease from the 22th day. The minimal quantity on the 29th day is remarkable, it practically means the total exhaustion of the TTP. According to the curve denoting the

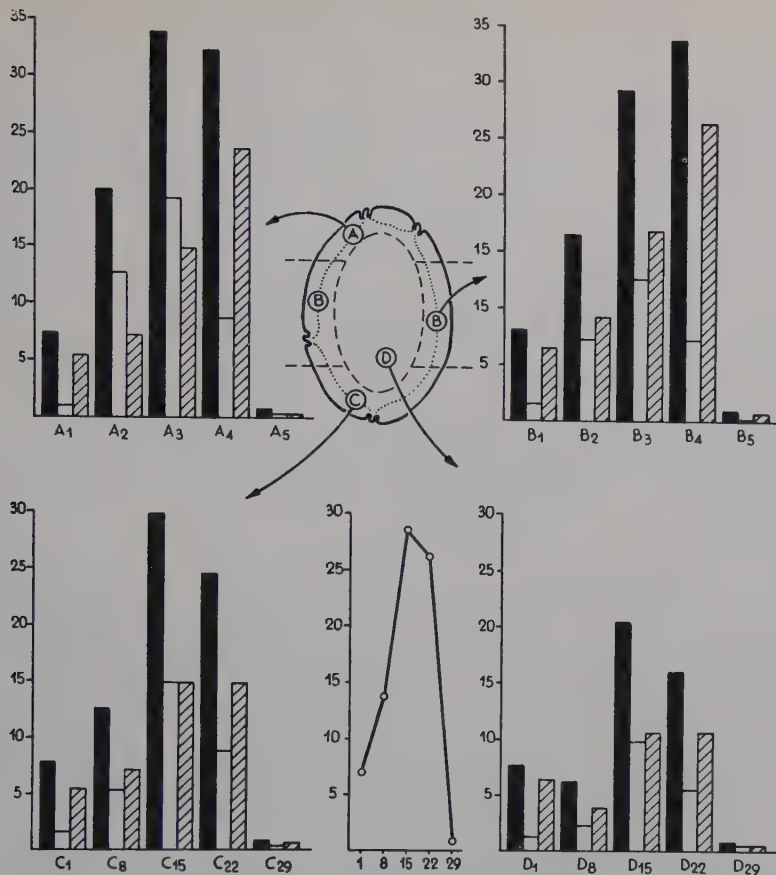


Figure 1. Changes of the quantity of bound (white), free (marked with lines) and of total tryptophane (black) in the tubers of "Kisvarda rose" forced with rindite. In the centre, above are the longitudinal-section of the tuber and the analysed sectors (A, B, C, D); below are shown the average tryptophane contents of all the sectors. 1, 8, 15, 22 and 29 denote the number of days following treatment. On the ordinates mg/100 g.

average TTP contents of the sectors (Figure 1, in the middle, down) the highest TTP contents of the tubers is on the 15th experimental day, it remains still high on the 22th day but afterwards it soon decreases.

Discussion

While analyzing the metabolism of the TTP, the changes of the TTP contents in the spontaneously sprouting (6) and rindite treated tubers were observed, further, the changes of the TTP level of tubers of different ages

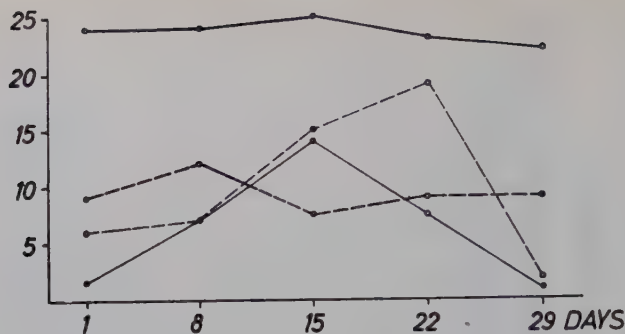


Figure 2. Changes of the bound and free TTP contents in the spontaneously sprouting tubers and in those forced by rindite. On the ordinate mg/100 g.

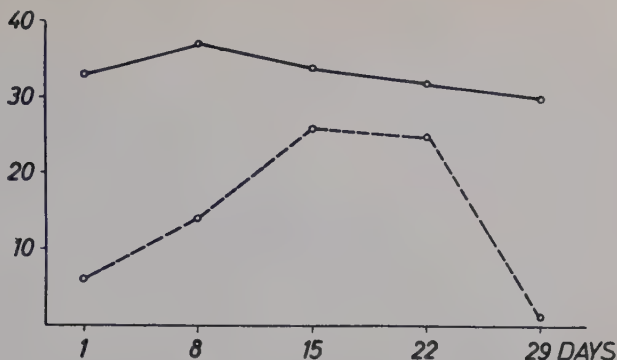
— bound TTP in the spontaneously sprouting tubers
 - - - free " " " " " " " "
 — bound " " " tubers forced with rindite
 - - - free " " " " " " " "

compared and, finally, the relation between the TTP-level and the appearance of the IAA in the sectors (9) and in the different phases of the sprouting examined.

As a precursor of the growth regulating indole compounds, mainly the free TTP can be taken into consideration, so this had to be regarded first of all when studying the rate of development of the buds. The changes in the bound TTP was not of direct concern as it may be assumed that the decrease of its quantity observed during the sprouting — in several cases significant — is connected with the formation of free TTP. Anyhow, the decrease of the quantity of the bound TTP in the different sectors may be attributed to its release as free TTP, partly decomposed to products with properties of growth substances, partly carried into other parts of the tuber where it is stored as free TTP, or is used again in secondary syntheses.

The free TTP content in the sectors "A" and "C" of the spontaneously sprouting tubers follows a double peaked curve, in sector "B" it decreases from the beginning, while in "D" the decrease occurs after a temporary increase (Figure 2). In the tubers treated with rindite it increases in all the sectors up to the 22th day of the experiment, then suddenly disappears. In the spontaneously sprouting tubers the bound TTP content is higher in every sector and in every phase of the sprouting than that of free TTP, while in the rindite treated tubers the quantity of the free TTP exceeds that of the bound TTP, except on the 8th and 15th days of sector "A" (Figure 2).

Figure 3. Total TTP contents in different phases of the sprouting, in the spontaneously sprouting tubers of "Kisvarda rose" (unbroken line) and in the same treated with rindite (dotted line). On the ordinate mg/100 g.



It is to be mentioned, however, that in spite of this the content of free TTP in the tubers treated with rindite is hardly higher than in the spontaneously sprouting ones, but the quantity of the bound TTP is essentially lower (Figure 2).

As to the total TTP contents (Figure 3) there is a very marked difference between the tubers of the two different ages.

It is evident from the figures that the increase and decrease of the TTP level are more intensive and the changes are quicker in the tubers treated with rindite than in the spontaneously sprouting ones.

Irion and Fischnich (1952) also noted the parallel increase of the TTP content on the one hand with the rise of the rindite concentration and on the other with the advance of the sprouting.

The tryptophane metabolism described in the introduction justifies the comparison between the quantitative changes of TTP and IAA (Varga and Ferenczy 1957, Szalai 1959 b) in the different sectors observed during the sprouting (Figure 4).

The quantity of free TTP in sector "A" increases quickly and uniformly from the beginning of the experiment up to the 22th day, then almost totally disappears until the 29th day. At the same time the quantity of IAA is constant and small from the 1st to the 8th day, rapidly rises to the 15th day, then slowly but constantly decreases. On the 29th day the amounts is three times the initial value.

The change in the content of the free TTP during the sprouting in sector "B" is in agreement with that in sector "A", its relation to the IAA is, however, different. The IAA content is considerably lower, its rise is slower but its level is higher on the 29th day, than in sector "A". In the sector "B" the free tryptophane rises only to the 15th day, later decreases and on the 29th day has almost disappeared. The IAA curve in this sector bears resemblance to that of sector "B".

tubers is unexpected and somewhat surprising. The formation of the approximately identical level in different sectors of the spontaneously sprouting tubers indicates that the required quantity of free TTP is constantly released from the bound TTP for the formation of the growth substances, and this is directly utilized; the rate of release of bound TTP is determined by the utilization.

5. In the tubers treated with rindite the changes of the free TTP content indicate more strikingly the relation between TTP and IAA metabolism than those in spontaneously sprouting tubers.

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Induction Phenomena in Photosynthesis. Simultaneous Measurements of CO₂ and O₂ Exchange

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In most studies on photosynthesis the authors are content with expressing the rate of the process in terms of either carbon dioxide taken up or of oxygen formed. It is reasonable to assume that several sources of inaccuracy for the interpretation of the results might be reduced by use of a measuring technique permitting simultaneous determination of both of these metabolic components.

The diaferometer method (the gas thermal conductivity method) which, in addition to other advantages, permits a continuous determination of the changes in the composition of the experimental gas has so far allowed the determination of the $-\Delta\text{CO}_2$ only. Attempts to increase the sensitivity of the apparatus, in order to make it possible to determine the ΔO_2 as well, failed due to the fact that the difference between the thermal conductivities of oxygen and nitrogen, which constitutes the main part of the atmospheric air, is but slight. Compared to the corresponding difference between the thermal conductivities of carbon dioxide and atmospheric air this latter difference is found to exceed the former by a factor of about ten. Hence the diaferometer method is not feasible in practice for the amounts of oxygen concerned in cases where it is judged appropriate to use only 0.25 to 1 g. of plant material (Vejlby 1958 a). On the other hand this great difference in sensitivity means that the measurements made by the diaferometer method in a mixture of carbon dioxide and atmospheric air may be directly used as an expression

of the $-\Delta\text{CO}_2$. The correction theoretically to be made on account of the simultaneous change in the oxygen content of the air is so slight as to be of no practical importance. The present author therefore ignored it in his previous studies on the photosynthetic induction of the mosses (Vejlby 1958 a, b).

Butler (1957) showed that the difficulty in measuring O₂ exchange might be overcome by substituting helium for nitrogen. In a gas mixture containing 3 per cent. of CO₂, 20 per cent. of O₂, and 77 per cent. of He, the differences between the thermal conductivities of oxygen versus the mixture and those of carbon dioxide versus the mixture, as expressed as per cent. of the total thermal conductivity, are 80 and 88 respectively. The corresponding figures for oxygen and carbon dioxide in atmospheric air are 4.5 and 40. In other words: while the difference between the thermal conductivities of atmospheric air and oxygen is 0.1 and between those of atmospheric air and carbon dioxide 0.9, the corresponding difference between "helium air" and oxygen and between "helium air" and carbon dioxide are 9.2 and 10.1, respectively.

When helium is substituted for the nitrogen of a gas mixture saturated with water, only the three components He, O₂, and CO₂ can influence the measurements. As the difference between the thermal conductivities of these components is sufficient, and as helium does not participate in the photosynthetic process, it will be possible in one set of matching measuring ducts of a diaferometer to directly determine the value of $(-\Delta\text{CO}_2 + \Delta\text{O}_2)$ while simultaneously a determination of ΔO_2 is made in another set of measuring ducts, in which the gas from the plant chamber is freed of carbon dioxide in advance (cp. Vejlby 1958 a, Figure 1). The value of $-\Delta\text{CO}_2$ is then computed on the basis of these determinations.

In previous studies (Vejlby 1958 a, b, c) time curves depicting the variation of $-\Delta\text{CO}_2$ for the moss species *Polytrichum attenuatum* were found to show a peak 30 to 60 seconds after the onset of illumination. This peak has been shown to develop very distinctly in a gas mixture containing 3 per cent. of CO₂ at a temperature of about 15°C. or below, whereas it disappears when the temperature is raised to 30°C. Finally it has been shown that if the light is turned off at the time when the peak mentioned is fully developed or a few minutes later, the time curve descends below the axis of abscissa. In other words, under circumstances such as these a gush of CO₂ is registred.

In the present study continuous determinations were made of CO₂ and O₂ exchange in a moss species and in crowberry (*Empetrum nigrum*) in two types of experiments, one in which the plants were illuminated continuously for 20 minutes, and another in which a 4 minute dark period was inserted after one minute of illumination and before resuming illumination for 15 to 20 minutes. These experiments were made at a temperature of 15°C., and

finally experiments were made also with continuous illumination of moss plants at 30°C. The aim was to study the mutual variation of the CO₂ and the O₂ time curves under these different experimental conditions in order to further increase our understanding of the induction phenomena during photosynthesis.

Material and Methods

As was the case in previous studies, *Polytrichum attenuatum* Menz. from Rude Forest or Tisvilde Plantation was used as a sample plant. In some few experiments were used shoots of crowberry (*Empetrum nigrum* L.) from Gudmindrup Lyng in Northwest Zealand. As before the plants were kept in covered glass containers in an air thermostat and were from time to time supplied with sufficient water. The light intensity during storage was kept as in previous work, but the illumination was adjusted in such a way that 18 hours of light were followed by 6 hours of dark. During the light period the temperature was 10°, during the dark period it was reduced to 5°C. For each experiment plant material corresponding to a fresh weight of 0.25 g. was used. In the case of *Empetrum* this corresponded approximately to 3 top shoots of a length of 4 cm.

The experimental arrangement was principally as before. At first an attempt was made to obtain an experimental gas containing 3 % of CO₂, 20 % of O₂ and 77 % of He by injecting a mixture of 13 % of CO₂ and 87 % of O₂ from a steel cylinder into a stream of He at a ratio of 23 : 77. This technique, however, which has previously been successfully used to mix CO₂, O₂ and N₂ at varying ratios proved to be inapplicable in the present case. It was not possible to produce a gas mixture stream of sufficiently constant composition, probably due to the great difference in specific gravity of the components. Hence it proved necessary to mix all three gases in advance in a steel cylinder under a pressure of 80 to 100 atm. and to use this mixture as the gas source.

During the experiments two time curves were drawn simultaneously. One represents the changes in the CO₂ and O₂ content of the gas stream determined jointly with one set of measuring ducts, the other exclusively representing the changes in O₂ concentration measured by another set of measuring ducts after the gas stream has passed a CO₂ trap. The sensitivity of the apparatus towards the changes of the gas mixtures in the two set of measuring ducts differs for several reasons. For one thing the thermal conductivities of the gas components are different, as mentioned, secondly a certain dissimilarity of the mechanical arrangement of the ducts is to be expected, and finally, the sensitivities of the two galvanometers are different. In order to adjust the CO₂ and O₂ curves to the same basis line it was assumed that the photosynthetic quotient ($\Delta\text{O}_2/-\Delta\text{CO}_2$) obtains a value of 1.0 when the induction phase is finished and photosynthesis has reached its steady state. Numerous measurements of steady state photosynthetic quotients for varying kind of plants (cp. Rabinowitch 1945, Table 3.I and Arnon 1955) show values very close to unity and tend to make this assumption fully justifiable.

The Figures 1 and 2 may serve as examples of the mode of computation. After having been placed in the experimental gas stream in the dark for 1 hour, the moss plants were illuminated for 20 minutes, and the galvanometer readings recorded

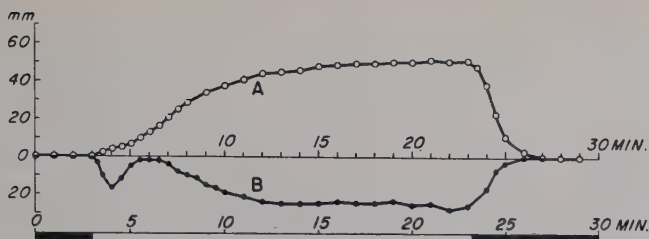


Figure 1. Time curves of photosynthesis for 0.25 g. of moss plants in a helium atmosphere (3 % CO₂ and 20 % O₂ in He). Temperature 15°C. Curve A: ΔO_2 . Curve B: $-\Delta(CO_2 - O_2)$. Light and dark periods are indicated by light and dark areas under the curves. Abscissa: time in minutes. Ordinate: galvanometer reading in mm.

On this basis the curves A and B were drawn (Figure 1), representing the changes in the oxygen content of the photosynthetic gas (curve A) and the combined changes for oxygen and carbon dioxide (curve B). Due to the fact that a reduction of the CO₂ content of the air causes an increase of its thermal conductivity, whereas an increase of its O₂ content causes a decrease, galvanometer readings resulting from a simultaneous uptake of carbon dioxide and a formation of oxygen of the same order of magnitude will be lower than readings based on the formation of oxygen exclusively. Consequently the readings plotted in curve B after the conclusion of the induction period are considerably lower than those registered at the same time in curve A. Hence curve A represents ΔO_2 and curve B $-\Delta(CO_2 - O_2)$. By means of these two curves a third curve (C) then was constructed depicting $-\Delta CO_2 = -\Delta(CO_2 - O_2) + \Delta O_2$. Due to the facts previously mentioned it is not possible directly to compare this curve to curve A; but assuming that $\Delta O_2 / -\Delta CO_2 = 1.0$ when steady state photosynthesis sets in (in this case 21 minutes after the beginning of the experiment) a correction factor (in this case 1.51) may be computed, by means of which the course of curve A is recalculated. The result is named curve D, and the final result of the experiments appears from curves C and D in Figure 2.

Results

The first series of experiments concerned the course of the CO₂ and the O₂ time curves at continuous illumination of moss plants in a helium atmosphere containing 3 % of CO₂ and 20 % of O₂. The result appears in Figure 2 showing the uptake of carbon dioxide (curve C) to start simultaneously with illumination and to reach a tentative maximum within 1 minute; it then decreases sufficiently to show the well known induction peak. Its increase thereupon continues until the induction phase is finished after approximately 12 minutes of illumination. Likewise, the formation of oxygen (curve D) sets in simultaneously with the onset of illumination, but in this case the increase is even, the time curve showing no peak, and after two minutes of illumina-

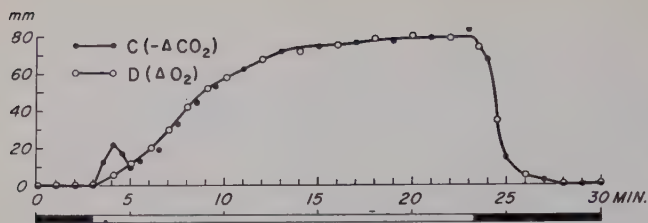


Figure 2. Time curves for 0.25 g. of moss plants in a helium atmosphere. The curves computed on the basis of the curves of Figure 1.

tion the two curves, C and D, are following the same course. The experiment has been repeated over and over with identical results.

The carbon dioxide time curve has previously been shown to descend below the axis of abscissa if the illumination is discontinued during the period in which the induction peak is at its maximum, or in other words, under such circumstances a CO_2 gush is registered (Vejlby 1958 b, c). In order to study the course of the oxygen production under these conditions, similar experiments were made in which the light period was interrupted after one minute, and in which both the CO_2 and the O_2 curves were determined. Figure 3 represents an example of the results, showing an experiment in which the moss plants were illuminated for 1 minute, then darkened for 4 minutes, and finally illuminated again for an additional 20 minutes. The inserted dark period sets in 15 seconds after the induction peak has been fully developed. The carbon dioxide curve (Figure 3 C) confirms what has been previously observed, demonstrating with certainty a liberation of carbon dioxide in the inserted dark period as well as a development of a secondary peak or shoulder appearing 45 to 60 seconds after the onset of the

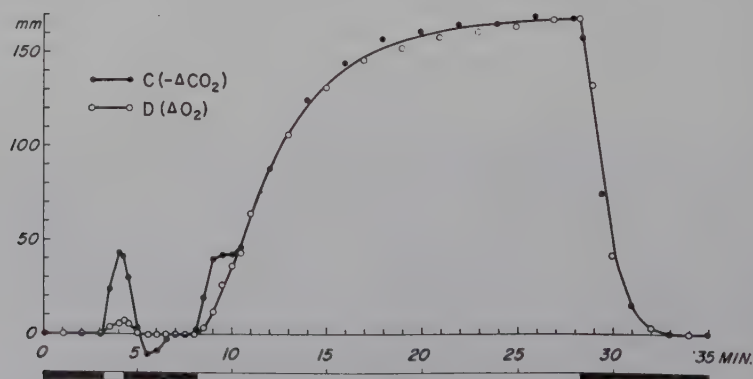
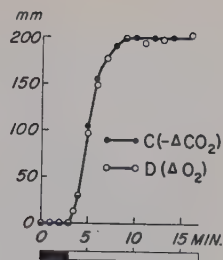


Figure 3. Time curves for 0.25 g. of moss plants in a helium atmosphere (3 % CO_2 and 20 % O_2 in He). Temperature 15°C . 1 minute of light and 4 minutes of dark followed by light.

Figure 4. Time curves for 0.25 g. of moss plants in a helium atmosphere at 30°C.



secondary illumination period (cp. Vejlby 1958 b, Figures 2 and 3). The oxygen curve (Figure 3 D of this paper) is also developed immediately after the onset of illumination, increasing more slowly than the CO₂ curve during the first short light period for, during the dark period, to descend to the axis of abscissa and stay at zero until the light is again turned on. It then increases immediately, but the increase is smaller than the increase of the CO₂ curve at the same time. Not until the shoulder of the CO₂ curve has been settled do the two curves become congruent and stay so for the remainder of the experimental period.

The experiments described so far were made at 15°C. As the peak of the CO₂ curve has been previously shown to be no longer registrable when the temperature is raised to 30°C. (Vejlby 1958 a, Figures 5 and 6), additional experiments were carried out at this temperature by means of the new helium technique. A sample result appears from Figure 4. It shows the two time curves for the uptake of CO₂ and for the formation of O₂, respectively, to be congruent throughout the induction phase, and no induction peak can be registered. Duplicate experiments gave similar results.

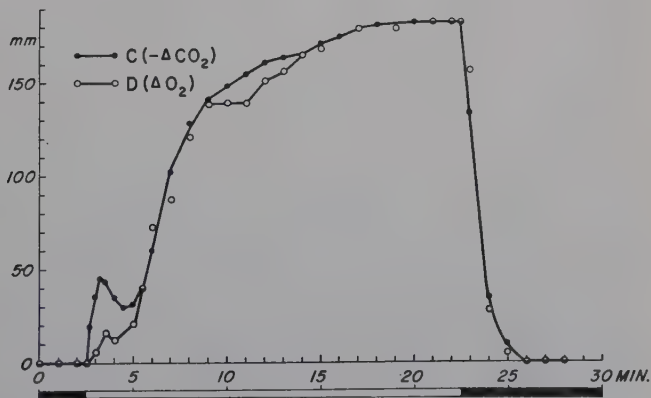


Figure 5. Time curves for crowberry (3 top shoots, length 4 cm., total fresh weight 0.25 g.) in a helium atmosphere at 15°C.

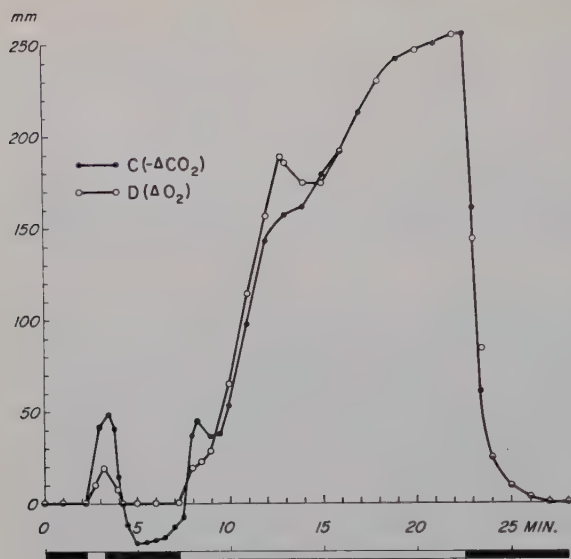


Figure 6. Time curves for 0.25 g. of crowberry. A 4 minute dark period inserted following 1 minute of light.

Finally additional experiments were made with material from a systematically higher plant, viz. crowberry shoots. In this case the results are found to deviate somewhat from those of the moss experiments. Figure 5 shows an example of the results obtained with crowberry leaves. In principle the course of the CO_2 curve is like that known from the experiments with *Polytrichum*, but the O_2 curve differs in two respects. In the first place a peak occurs in the crowberry curve after 1 minute of light. Secondly this curve, after having followed the course of the CO_2 time curve for some time, after 6 minutes forms a distinct shoulder, where the formation of oxygen apparently remains constant for two minutes. Subsequently the curve increases again, and after a total illumination period of 11 minutes it is reunited with the CO_2 curve, and the two time curves now follow identical courses for the remainder of the experimental period.

In order to check the reaction of the two crowberry time curves to an interruption of the illumination at the very moment when the primary peaks have reached their maximum value, an additional experiment was carried out corresponding to the one with *Polytrichum* recorded in Figure 3. Its results are seen in Figure 6. After standing in the dark in the gas stream (3 % of CO_2 , 20 % of O_2 , 77 % of He) for one hour, shoots of crowberry were illuminated for 1 minute, darkened for 4 minutes, and re-illuminated for 15 minutes. As in previous experiments the dark interval causes the CO_2 time curve to descend below the axis of abscissa, thus registering an libera-

tion of carbon dioxide. When the light is again turned on, a secondary peak is formed on the CO₂ curve in agreement with previous moss experiments. The O₂ time curve decreases after darkening of the crowberry leaves to the axis of abscissa and remains there until the conclusion of the dark period. In this respect the curve behaves as the moss O₂ curves. During the secondary light period the O₂ curve increases fairly evenly together with the CO₂ curve until, after 6 minutes, another peak is formed corresponding to the shoulder seen in curve D of Figure 5. Approximately two minutes after this secondary peak of the O₂ time curve has reached its maximum, the oxygen and the carbon dioxide curves again become congruent and stay so for the remainder of the experimental period.

Discussion

It has been mentioned that Butler (1957) introduced the use of helium atmospheres in diaferometer studies of photosynthesis. In the same work a number of CO₂ and O₂ time curves for *Hydrangea* leaves were published, determined in a helium atmosphere containing 4 % of CO₂ and 20 % of O₂. The curves differ from the present ones by being apparent photosynthesis curves. In most of these experiments the temperature was about 0°C., but in one case it was room temperature. The latter is the one which may to the greatest advantage be compared with the present results; Butler here registers first a CO₂ curve which in principle agrees with those found by others and secondly an O₂ time curve which throws new light on the induction phenomena. This curve confirms the view that the formation of oxygen commences immediately upon the onset of illumination of the plant material, as does the uptake of carbon dioxide; furthermore the curve is remarkable by forming a shoulder during the first minute of the light period and not until later increasing evenly. In the experiments at 0°C. Butler registers not only a shoulder in the O₂ curve, but an actual peak which develops most distinct when the previous dark period has lasted 10 minutes. It is still fairly distinct when the dark period lasts for 1 minute only, but disappears completely when it increases to 40 minutes.

The O₂ curves shown in the present work differ from those of Butler's in several respects. In the moss experiments no form of peak or shoulder could be demonstrated to exist during the first minutes of photosynthesis. On the contrary, the curves show an even, uninterrupted increase throughout the induction period (Figures 2, 3, 4). Not so in the crowberry experiments, in which case in addition to a primary peak formed during the first minute of illumination a secondary shoulder or peak occurs after approximately 6 minutes of illumination (Figure 5, 6). The latter reaction has not been

registered by Butler, and the primary peak only occurs as such in his experiments at 0°C ., while at room temperature only a shoulder is observed. The development of a peak in Butler's curves at 0°C . apparently takes place only under very special conditions, since, as mentioned, a previous dark period lasting 10 minutes is required. But in the present experiments this reaction has been registered at 15°C . after a previous dark period of 1 hour, and a 4 minute dark period only seems to produce a slight shoulder (Figure 6 curve D). The secondary peak occurring in the same curve corresponds to the one also existing in the case of continuous illumination (Figure 5 curve D).

The CO_2 time curves of Butler do not seemingly add anything new to our knowledge concerning the primary induction peak, but in the experiments at 0°C . he registers an evolution of CO_2 when light is turned off after the conclusion of the induction phase. A similar reaction was observed by van der Veen (1949) in experiments carried out in a gas mixture containing 97 % of H_2 and 3 % of CO_2 , and in this laboratory it has proved possible to induce it during experiments in ordinary atmospheric air containing 3 % of CO_2 by poisoning the plant material with iodoacetamide. But it has not been possible to observe this reaction in normal plants which have been studied in atmospheric air as well as in helium-oxygen mixtures, both with 3 per cent. of carbon dioxide added.

Butler endeavours to explain his data on the basis of the assumption that particularly at low temperatures an interaction might take place between photosynthesis and certain intermediates of the respiratory processes, in particular phosphoglyceric acid. Although this assumption cannot be rejected with certainty, more recent studies have suggested that the chloroplasts themselves have no respiration proper, and that the localization of photosynthesis and of respiration is spaced relatively far apart in the cells (Arnon 1955, James & Das 1957, cp. Steemann Nielsen 1955).

A consideration of the present data show the moss experiments to give the clearest picture. The oxygen curves suggest that during all of the induction period the photosynthesis proper increases evenly, probably due to the steadily increasing amounts of the carbon dioxide acceptor, ribulose diphosphate. The occurrence of a peak in the CO_2 curve may perhaps most readily be explained by assuming the existence of yet another acceptor of carbon dioxide. Naming this acceptor I (its effect being the first to show up following darkening) and ribulose diphosphate acceptor II, it may be assumed that acceptor I, the absolute concentration of which never amounts to very much, yet at room temperature, is present or formed the very moment light is turned on, in an amount to make it preponderant over acceptor II. As with time the latter is synthesized in greater amounts it completely takes over the uptake of carbon dioxide, and the CO_2 initially bound by acceptor I is trans-

ferred to acceptor II during the first few minutes. In this way it also becomes possible to explain the CO₂ gush registered when a dark period is inserted at the very moment when the primary peak is at its maximum (Figure 3 and 6 cp. Vejlbj 1958 b), the quantity of CO₂ bound by acceptor I might then simply be given off immediately the light is turned off. At higher temperatures (30°C.) where the peak of the CO₂ time curve disappears, the formation of acceptor II simply takes place at such a rate as to completely overshadow the function of acceptor I; this appears to be probable according to Figure 4 of this paper.

In the case of *Empetrum*, the O₂ time curves seem to indicate that the induction phenomena are of a more complicated nature. Here peaks occur in the CO₂ as well as in the O₂ curves. This difference, however, may not be attributable to the induction phenomena proper, but may be due to anatomical differences between *Polytrichum* and *Empetrum*. Whereas the photosynthetic organs of the mosses are thin, rich in chlorophyll, and without stomata, the leather-like leaves of *Empetrum* are fairly complicated as to gas exchange. The stomata are found only on the underside, and this underside is even closed in, the leaves being rolled up and the cleft between the two leaf edges being densely overgrown with hairs. These facts may possibly affect the gas exchange so as to give the appearance of extremely complicated processes during the induction phase. In particular it seems to affect the oxygen curve causing not only a peak at the beginning of the illumination period, but also an additional secondary peak after approximately 6 minutes of illumination. However, the elucidation of these phenomena so different from those pertaining to the mosses would involve additional experimental work which it has not been possible to carry out in connection with the study here described.

Summary

The initial uptake of carbon dioxide and the formation of oxygen during photosynthesis in the moss species *Polytrichum attenuatum* Menz. and in crowberry leaves (*Empetrum nigrum* L.) were studied by means of the diaferometer method using gas mixtures of helium, oxygen, and carbon dioxide.

In the case of the mosses the O₂ time curve shows photosynthesis to increase evenly throughout the induction period, probably due to the formation of steadily increasing amounts of the carbon dioxide acceptor, ribulose diphosphate. The CO₂ time curve suggests the presence during the first minute of photosynthesis of yet another carbon dioxide acceptor. A suspen-

sion of the illumination after 1 minute causes this acceptor to release the quantity of CO_2 taken up, whereas a continuation of the illumination appears to cause the carbon dioxide to be transferred to ribulose disphosphate and hence to the photosynthetic reaction.

In the case of crowberry leaves the data, at least the O_2 time curve, suggest that the induction phase in higher plants is of a somewhat more complicated nature than that found in mosses, but the two carbon dioxide acceptors, assumed to be present in the moss plants, appear to occur here as well.

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A Simple Laboratory-Made Turbidimeter

By

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In laboratories one often needs a handy instrument for measuring the relative turbidity of solutions or fluids, e.g. for studying the growth of micro-organisms or following the action of pectin degrading enzymes in fruit juices.

Even though there are several commercial turbidimeters or nephelometers available, the author would like to draw attention to a simple set-up, which, using a special principle, is very easy to construct and still quite accurate and sensitive. In comparison with several other instruments it has the advantage that the value obtained is practically independent of the colour of the fluids. The main principle is not new, but it has here been simplified as much as possible especially by the use of cylindrical cuvettes or test tubes. Thereby it has also become particularly suitable for serial analyses.

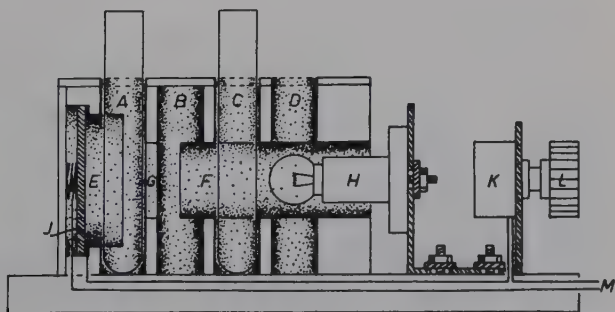


Figure 1. *Semi-schematic longitudinal section through the turbidimeter.*

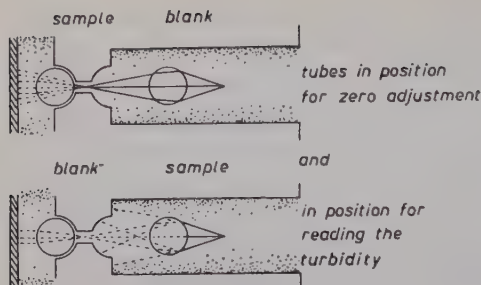


Fig. 2.

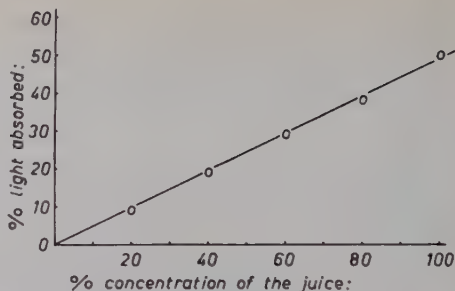


Fig. 3.

Figure 2. The working principle of the instrument.

Figure 3. Diagram showing the relation between turbidity, as measured by the instrument, and concentration of fresh apple juice.

Construction: The prototype was made to hold the "EEL" matched colorimeter tubes with a diameter of 11 mm., which determines the size dimensions.

In a wooden block, 5×5×8 cm., there are made four holes fitting the tubes, i.e. A, B, C and D in figure 1. From each end there are further made two bigger holes, E and F. Between the holes A and B there is taken up a 4 mm. broad vertical slit, G.

To the right, in the hole F, there is mounted a small electrical lamp, H, supplied by some constant energy source. To the left a common selenium cell, J, is arranged. The connections from the Se-cell are brought via a potentiometer, K (5.000 ohms), adjustable by the knob L, to an external galvanometer attached at M (in this case a "Philip's Universal Measuring Instrument" was used).

Principle: Both the blank and the sample to be measured are kept in the instrument at the same time as illustrated schematically in figure 2. The zero adjustment is first made with the sample next to the Se-cell. The blank then acts as a cylindrical lens focusing the lamp filament in the slit G. In the sample the light is dispersed due to the turbidity, but relatively much of the dispersed light hits the Se-cell.

When the galvanometer has been adjusted to maximum deflection at the end-point of the scale, here called "zero", the tubes are exchanged so that the blank comes next to the Se-cell. Then only a minor fraction of the dispersed light will pass the slit G and hit the cell. The deflection of the measuring instrument will decrease, and this decrease will be proportional to the turbidity of the sample.

In figure 3 is shown the result of a simple dilution experiment using turbid apple juice diluted with water. The turbidity, expressed as the percentage

of light absorption when changing the tubes, is linearly proportional to the concentration of the juice.

It is easily understood that the value obtained will be independent of the colour of the sample, which is indeed very important. As blue light undergoes more dispersion than red, it might be wise to use a colour filter for more careful work. A narrow filter might be inserted in the hole B, e.g. Known amounts of some dye might also be added to the blank.

It need perhaps not be mentioned that the instrument might then also be used as a simple colorimeter without any changes.

Summary: The paper contains a description of a simple turbidimeter, which can easily be assembled in the laboratory workshop for a small outlay.

Chemical Analyses of Ripening Peas

By

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Ripening seeds, especially of species belonging to the Leguminosae family, constitute a classic experimental material for the study of the synthesis of starch, protein, etc. in the plants. In an earlier investigation (1) the author has shown that in seeds of *Pisum sativum* the sugar concentration in the water phase of the seeds is constant during the greater part of the ripening process. Furthermore it could be demonstrated that the starch was synthesized in two different steps, first slowly and towards the end of the ripening process much more rapidly.

These investigations have been continued, and they give in the results reported in this paper above all a picture of the distribution between monosaccharides and compound sugars in seeds and hulls during the ripening process. In addition a method has been worked out for the preservation of the plant material.

1. Sugar and Starch Analyses of Preserved Plant Material

During the relatively rapid ripening process, when the most interesting changes from a chemical point of view take place, it may be difficult to perform all the desired analyses. A rapid and simple method of preservation would therefore be of value.

McKee *et al.* (2) give a method for preservation of peas for subsequent chemical analysis. The fresh seed material was placed in ethanol in cans which were thereafter seamed. It was assumed that the moisture content of

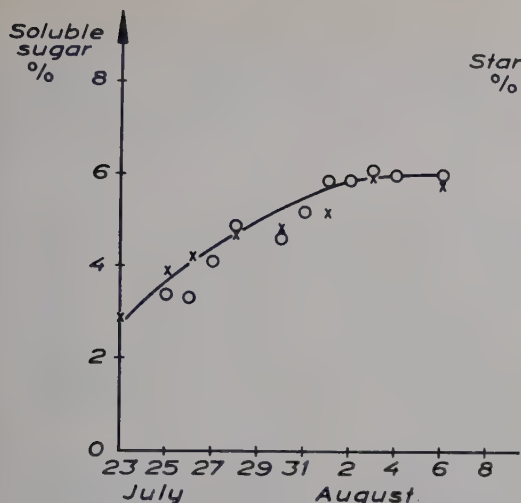


Fig. 1.

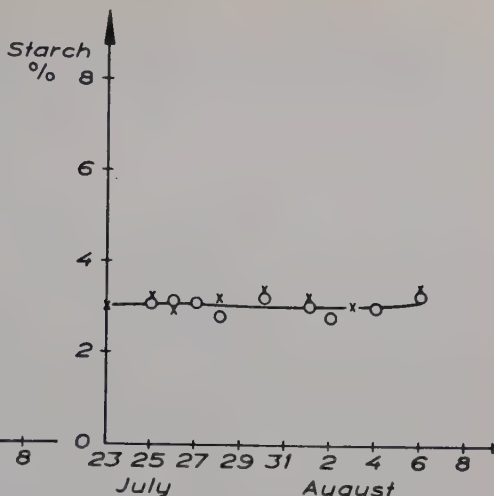


Fig. 2.

Figure 1. Sugar content in per cent of fresh weight in peas during ripening.

x=fresh seeds

o=preserved seeds

Figure 2. Starch content in per cent of fresh weight in peas during ripening.

x=fresh seeds

o=preserved seeds

the seeds was 80 per cent, and the addition of alcohol was chosen so that the final concentration was 75 per cent.

This method in a somewhat modified form has been used in an investigation of the sugar and starch contents in ripening peas.

Experimental

The material investigated was peas of the Profusion variety, which were harvested at the end of July and the beginning of August 1956. The preserved material was first analyzed in January—February 1958. The pods were harvested in a field close to the laboratory. Only pods in the same developmental stage, *i.e.*, from the same node, were used (1). Approximately one hour after the harvesting the peas were separated from the hulls and the analyses and preservation were begun.

Ten grams of plant material (seeds or hulls) were ground in a small Waring Blender with 40 ml 96 per cent ethanol for 3 minutes. The mixture was transferred to an enameled can (73×54 mm). Washing was performed with 96 per cent ethanol to a final volume of c. 100 ml. Thereafter the can was sealed and stored at room temperature.

Immediately after the arrival of the peas at the laboratory the weight of 100 seeds, moisture content, sugar and starch were determined on the fresh material. The analytical methods employed have been previously described (1).

Results

The comparison between the results obtained in the determination of sugar and starch in the fresh peas and in such which had been preserved in ethanol for 18 months is shown in Figures 1 and 2. The contents of soluble sugar and starch have been plotted against the time of harvesting. Only the first part of the ripening process has been studied, *i.e.*, before the sugar content decreases and the starch increases in the seeds (1).

The agreement between the analyses that were performed on the fresh and on the preserved seeds may be regarded as satisfactory. Corresponding experiments were carried out also on the fresh and preserved hulls. Even in this case the agreement was good for both sugar and starch.

2. Soluble Sugar in Seeds and Hulls during Ripening

The above described method of preservation has been used in an investigation of the distribution of monosaccharides and compound soluble sugars in seeds and hulls during the ripening process. The term "compound sugars" refers here to such which are hydrolyzed by hydrochloric acid. According to Bisson and Jones *c.* 95 per cent of the soluble sugar in peas is composed of sucrose (3). These results have been recently verified by chromatographic investigations of Turner *et al.* (4), who could not ascertain the occurrence of other di- or oligosaccharides. By the determination of reducing sugar with Fehling's solution in the alcoholic extract of pea seeds before and after hydrolysis with hydrochloric acid, one can thus obtain a quantitative measure of the sucrose content. According to Turner *et al.* (4) the monosaccharide fraction is composed of equal parts of glucose and fructose during the earlier stage of the ripening.

Results

The values found for dry substance, weight of 100 seeds, monosaccharides and sucrose are shown in Table 1. The analyses have been carried out on preserved material, stored for 18 months at room temperature.

On the basis of the above values, according to a previously described method (1), the sugar content, expressed in grams per 100 seeds, has been plotted against the moisture content of 100 seeds in Figure 3. From this

Table 1. *Concentration of sugars in ripening pea seeds, Profusion variety.*

Date of harvest	Fresh weight g/100 seeds	Dry substance % of fresh weight	Monosaccharides % of fresh weight	Sucrose % of fresh weight
23.7.56	8.1	16.2	0.7	2.7
	11.6	16.5	1.3	3.0
25	18.1	17.0	0.9	3.8
	19.5	17.1	0.8	3.8
27	19.2	17.9	0.9	4.0
	26.9	17.6	0.8	4.7
29	—	—	—	—
	25.2	18.0	0.9	4.3
31	38.5	19.0	1.2	4.7
	41.1	20.5	0.7	5.6
2.8.56	45.3	20.6	1.3	5.4
	61.2	21.4	1.1	5.7
4	51.0	21.3	1.1	5.5
6	69.1	21.8	—	—
	66.8	24.2	0.6	6.0
8	70.6	23.8	—	—
	70.9	25.4	0.6	5.6
10	79.8	26.2	—	—
	81.5	25.4	0.5	5.6
12	—	—	—	—
	81.0	24.2	0.3	4.8
14	88.8	28.0	0.4	4.8

Table 2. *Concentration of sugars in the hulls of ripening peas, Profusion variety.*

Date of harvest	Dry substance % of fresh weight	Monosaccharides % of fresh weight	Sucrose % of fresh weight
23.7.56	—	—	—
	15.9	3.1	0.9
25	17.3	3.3	0.9
	14.5	2.9	0.4
27	15.0	3.4	0.6
	14.7	3.7	1.1
29	—	—	—
	18.7	3.7	0.7
31	16.9	3.9	1.1
	18.0	3.0	1.0
2.8.56	16.1	3.3	1.4
	16.9	3.0	1.8
4	16.4	3.0	1.1
	—	—	—
6	14.7	3.4	1.0
	17.0	3.5	1.7
8	—	—	—
	15.3	3.3	1.7
10	17.4	3.5	0.9
	16.0	3.1	1.2
12	—	—	—
	15.7	3.1	1.1
14	18.3	3.7	1.4

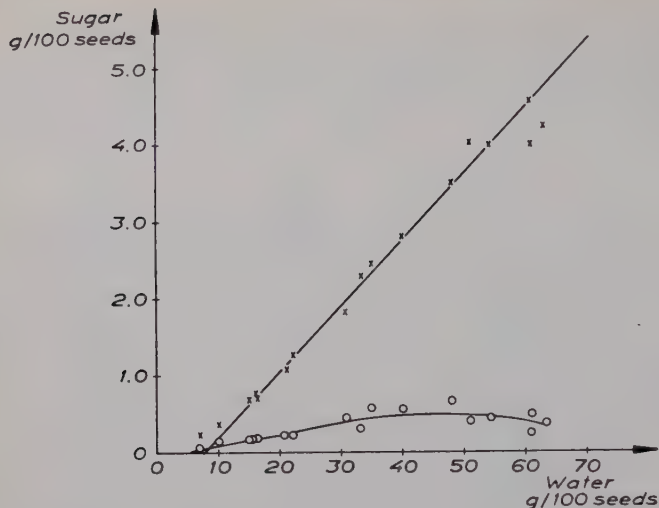


Figure 3. Sugar content as a function of water content in ripening peas:

x = sucrose

o = monosaccharides

figure it is clearly evident that the sucrose content increases linearly with the moisture content of the seeds during the ripening process. The content of monosaccharides increases only insignificantly.

The monosaccharides and sucrose were determined in the hulls of the same plant material (Table 2).

In Figures 4 and 5 the values from tables 1 and 2 have been plotted in such a way that the sugar concentration in the water phase of the seeds and of the hulls has been obtained as a function of the time.

Discussion

The method of preserving plant material described in this paper yields satisfactory results as far as the determination of sugar and starch are concerned. However the risks that the experimental material is changed by the preservation must not be underestimated (5), and in each individual case it is necessary to make careful comparisons by analyzing both fresh and preserved material. McKee *et al.* (2) did not grind their seed material. By grinding with alcohol it must, however, be expected that all enzymatic reactions are more rapidly inhibited.

Figure 3 verifies earlier experimental results (1, 6), which showed that the sugar content in the water phase of the seeds is c. 8 per cent. From figure 3 it is seen that the major part of the sugar is composed of sucrose. Figure 3 also shows that if the sucrose-water curve is extrapolated to sugar

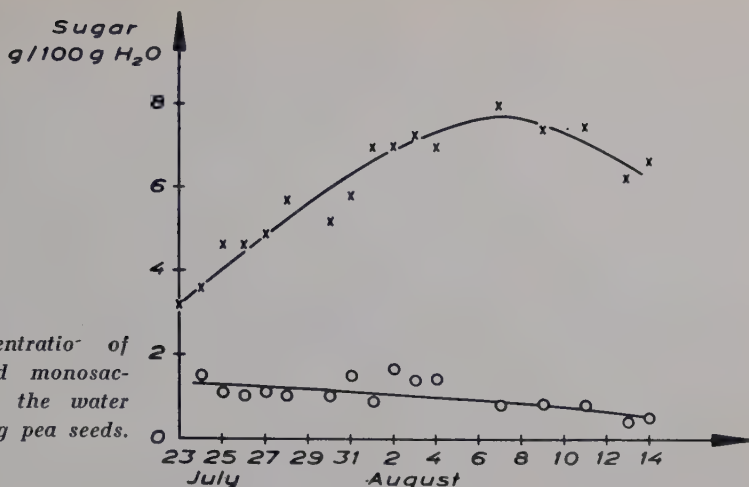


Figure 4. Concentration of sucrose (x) and monosaccharides (o) in the water phase of ripening pea seeds.

content=0, it does not pass through the origin. Thus there is a certain amount of water in the seeds which does not contain sugar and which has earlier been called "apparently free from sugar" (1). Investigations carried out in 1958 by the author have shown that this water occurs largely in the seed coats. If corresponding investigations are performed on cotyledons freed from seed coats, the curve will cut the abscissa closer to the origin. However, a small amount of "sugar-free water" is obtained even in this case. This problem will be treated in a later paper.

The comparison between the distribution of monosaccharides and sucrose in seeds and in hulls (Figures 4 and 5) shows that the total concentration of

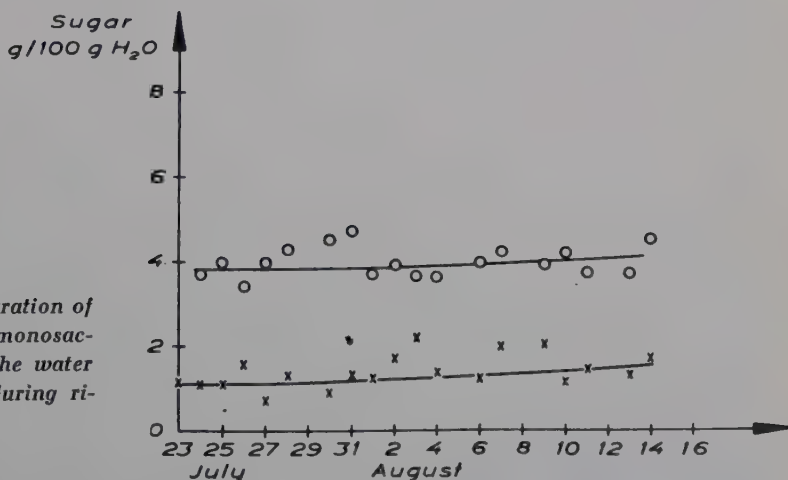


Figure 5. Concentration of sucrose (x) and monosaccharides (o) in the water phase of hulls during ripening.

sugar in the water phase is about the same as that obtained by McKee *et al.* (2). The sugar in the hulls is composed to c. 80 per cent of monosaccharides, whereas in the seeds monosaccharides comprise only 10—25 per cent. This verifies the results which Turner *et al.* (4) obtained by paper chromatography. They have only one value, determined about 25 days after flowering, which shows the distribution between sucrose and monosaccharides in the hulls. The present investigation shows that the distribution is about the same through the entire ripening process. Thus one cannot exclude the possibility that the carbohydrates are transported from the leaves and stored in the hulls in the form of monosaccharides, which later are taken up by the seed where they are converted to sucrose. The seed takes up monosaccharides from the hulls at such a rate that a sucrose content of c. 8 per cent can be constantly maintained in the water phase.

This investigation has been carried out with funds from Statens Naturvetenskapliga Forskningsråd.

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The Photosynthesis in Diploid and Tetraploid *Ribes satigrum*

By

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Several investigators have studied the morphological and anatomical differences between diploids and tetraploids, but very few have been interested in comparing the physiology of the diploids with that of the tetraploids. However, a certain amount of investigation has been done with the respiration and the photosynthesis.

In *Hordeum vulgare* Ekdahl (1941) found that the rate of photosynthesis was higher in the diploid than in the tetraploid, measured in mg. per g. fresh weight. In 1949 the same author showed that diploid *Galeopsis pubescens* had a lower rate of photosynthesis than the tetraploid, measured in mg. per cm.² of leaf area. But if he measured the rate of photosynthesis in mg. per g. fresh weight the diploid had the higher rate of photosynthesis. Stålfelt (1943) compared diploid and tetraploid *Trifolium repens* and found that the diploid had the lower rate of photosynthesis, measured in mg. per cm.² of leaf area. But — even in this case — the rate of photosynthesis in mg. per g. fresh weight or in mg. per g. dry weight was higher in the diploid than in the tetraploid. Andersson (1943), who made his investigation in *Hordeum vulgare*, found that the diploid had the higher rate of photosynthesis, measured in mg. per cm.² of leaf area or in mg. per g. fresh weight. Andersson, who measured whole plants, corrected his measurements after the more retarded growth of the tetraploid, but got in spite of this a lower rate of photosynthesis in the tetraploid.

Material and methods. In this investigation the photosynthesis has been studied in diploid and tetraploid *Ribes satigrum*. The diploid *Ribes satigrum*

Table 1. The apparent photosynthesis in diploid and tetraploid *Ribes sativum*, measured in mg. per 100 cm.² of leaf area and hour and in mg. per g. fresh weight and hour.

Mg. per 100 cm ² of leaf area		Mg. per g fresh weight	
Diploid	Tetraploid	Diploid	Tetraploid
7.21	6.62	3.33	3.14
8.08	7.37	3.42	3.37
6.13	4.63	2.79	1.92
6.64	6.15	3.14	3.11
6.70	5.66	3.33	2.71
7.77	5.94	3.88	2.87
6.89	4.36	3.70	2.14
7.46	6.33	3.73	2.80
8.06	6.21	3.82	2.40
6.64	6.12	2.74	2.54
8.11	6.78	3.09	2.60
9.24	9.62	3.96	4.08
9.66	7.96	3.90	3.47
6.79	5.76	3.08	2.46
9.08	7.17	4.18	3.14
8.44	6.60	3.68	2.74
7.26	6.10	3.33	2.44
6.61	6.21	2.98	2.53
6.17	4.95	2.81	1.96
9.09	7.83	4.32	3.50
8.37	6.35	3.35	2.35
160.40	134.72	72.56	58.27
M = 7.64 ± 0.23 t = 3.66***	M = 6.41 ± 0.25	M = 3.46 ± 0.093 t = 4.64***	M = 2.77 ± 0.115

is a F₁-hybrid between *R. sativum* and *R. nigrum*. The tetraploid is a colchicine-treated plant of the F₁-hybrid. The diploid is sterile, the tetraploid is fertile as is often the case with these allopolyploids. For further information see Nilsson (1955).

The photosynthesis has been determined by titration of Ba(OH)₂, according to the well-known vacuum-flask method. In each series of experiments 2 air-tests and 4 leaf-tests from the diploid and 4 from the tetraploid were taken. The leaves were taken from this year's shoot (1957) and were as far as an visual inspection could proof in the same state of development. The experiments were made at a temperature of 27°C and at a light intensity of 9780 lux. The time for each test was ±12 minutes. The means of each series of experiments are shown in Table 1.

A statistical treatment shows that the difference in rate of photosynthesis is highly significant between the diploid and the tetraploid whether the figures are made out in mg. per 100 cm.² of leaf area or in mg. per g. fresh weight. These results are thus in accordance with those of Andersson (*l.c.*).

In order to find out if the reason for the lower rate of photosynthesis in the tetraploid could be found in a lower chlorophyll content measurements

Table 2. *The chlorophyll content in diploid and tetraploid Ribes satigrum measured in mg. per 100 cm.² of leaf area.*

Diploid				Tetraploid			
Leaf nr	Chlorophyll a	Chlorophyll b	Carotenoids	Leaf nr	Chlorophyll a	Chlorophyll b	Carotenoids
1	3.72	1.47	0.66	2	3.38	1.69	0.82
3	4.02	1.74	0.62	4	5.11	2.35	0.83
5	3.52	1.34	0.67	6	3.83	1.35	0.68
7	3.37	1.40	0.72	8	4.58	2.05	0.77
9	3.84	1.60	0.64	10	4.85	2.13	0.78
11	3.68	1.57	0.59	12	5.51	2.58	0.74
13	4.49	1.91	0.74	14	3.50	1.52	0.61
	26.64	11.03	4.64		30.76	13.67	5.23
M = 3.81 ± 0.13				M = 4.39 ± 0.29			
1.58 ± 0.07				1.95 ± 0.16			
0.66 ± 0.02				0.74 ± 0.03			

$$t_a = 1.82 \text{ (} P < 0.05 \text{)} \quad t_b = 2.14 \text{ (} P < 0.05 \text{)} \quad t_c = 2.49 \text{ (} P < 0.05 \text{)}$$

of chlorophyll a, chlorophyll b and carotenoids were undertaken according to a method, described by Mc Kinney (1941). The results of these measurements are shown in Table 2. The tetraploid has a slightly higher content of chlorophyll a, chlorophyll b and carotenoids. The difference between the diploid and the tetraploid does not reach the level of significance. Thus there seems to be only a slight difference between the diploid and the tetraploid whether in the proportions of the different pigments or in the absolute weights of these pigments per cm.² of leaf area.

Györfy (1941), who compared the chlorophyll content of quite a lot of diploid and tetraploid dicotyledons, found that in each case the tetraploid had a higher chlorophyll content than the diploids per cm.² of leaf area.

As early as in 1918 Willstätter and Stoll showed that the chlorophyll content in normal leaves is far greater than is necessary for the photosynthesis. This explains why the slightly higher chlorophyll content is without consequence for the photosynthesis in these experiments.

Since thus the chlorophyll content could be excluded as a possible reason for the lower rate of photosynthesis in the tetraploid, the stomata of the leaf were counted. The method used was very simple. A piece of the epidermis was pulled from the leaf by a pincette and put into glycerine. The stomata were counted per range of sight of the microscope. A sample of 12—17 slides were taken from each leaf. Four leaves from the diploid and four from the tetraploid were counted. The means were as follows: The diploid 23.5, 26.6, 25.6 and 27.3. The tetraploid 16.3, 17.5, 15.6 and 21.6. The diploid thus has about 45 % more stomata per unit surface than the tetraploid. This means that the CO₂-uptake from plainly physical reasons must be less in the tetraploid.

It is a well-known fact that the tetraploids generally have larger cells than the diploids. Although the cell-size of diploid and tetraploid *Ribes satigrum* has not been measured in this investigation it may be concluded that the tetraploid has larger cells than the diploid from the fact that the tetraploid has fewer stomata per leaf area. A larger cell-size in the tetraploid means that the leaves of the tetraploid are a little thicker than those of the diploid (presuming the same number of cell-layers). That explains why the difference in photosynthesis between the diploid and the tetraploid is greater in the case when the measurements are made out per g. fresh weight than in the case when they are made per 100 cm.² of leaf area.

The slight difference in chlorophyll content might very well be explained by the fact that the figures are made out per cm.² of leaf area. Counting per g. fresh weight the difference between the diploid and the tetraploid ought to be less, because the tetraploid leaves are thicker.

Thus the doubling of the chromosome number causes in the tetraploid the larger cell-size and as a consequence fewer stomata, which in their turn cause less CO₂-uptake. The anatomical difference is thus the cause of the physiological difference.

Summary

A comparison has been made between diploid and tetraploid *Ribes satigrum* concerning the photosynthesis. In connexion with this the chlorophyll content has been measured, and the number of stomata has been determined both in the diploid and in the tetraploid. The results of this investigation show that the diploid has a higher rate of photosynthesis, a slightly lower chlorophyll content (counted per cm.² of leaf area) and about 45 % more stomata than the tetraploid.

The lower rate of photosynthesis in the tetraploid might be explained by the fact, that the tetraploid has fewer stomata per leaf area than the diploid. This means that the ultimate cause of the difference in photosynthesis is to find in the anatomical difference between the diploid and the tetraploid.

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Studies on a Shoot and Root Cell Elongation Stimulator in *Pinus silvestris*

By

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In an earlier investigation (Fransson 1953) are reported the results from an attempt to identify an auxin in *Pinus silvestris*. This auxin was obtained by extraction with ethyl alcohol of the two- and three-year-old parts of the stem and of the whorls of pine trees. The alcohol was evaporated from the crude extract and the auxin taken up in a water solution. This solution was purified by shaking with ether, firstly after addition of sodium bicarbonate, secondly after addition of tartaric acid. The acid ether fraction was transferred to agar, whereupon the auxin activity of the agar was measured by the *Avena* coleoptile curvature assay (Boysen Jensen 1937 and 1941).

The results obtained in this study showed the following character of the auxin from pine: 1. It has a stronger activity if an agar of pH 5.6 is used than in an agar of pH 2.7; on the other hand the activity of synthetic indole-3-acetic acid (IAA) was the same at these two pH-values (*cf.* however Dolk and Thimann 1932, Hemberg 1947); 2. The activity curve, *i.e.*, the relation between growth activity and substance concentration (expressed in the unit WAE), of pine auxin is different from that of IAA; 3. The diffusion coefficient of pine auxin through agar of pH 5.6 was obtained as 0.749, while a value of 0.583 was found for IAA. It should be emphasized that the diffusion coefficient of pine auxin is *nearly the same even after a purification of the substance by a diffusion through agar, so-termed double diffusion experiments.*

On the basis of these results it was suggested that the *pine auxin studied*

is not identical with IAA, but is some other auxin substance hitherto unknown.

In this study of the pine auxin, the chromatographic technique was not used. The purpose of the present investigation has therefore been to gather more information about the pine auxin by employing methods of paper chromatography. The plant material examined in this case was not obtained from trees but from young seedlings which were more easily available.

Experimental

Material and Methods

The pine material consisted of seedlings of *Pinus silvestris* of Northern Sweden type. They were grown in Vermiculite at room temperature and under long-day light conditions. When the seedlings were three weeks old after emergence, the green shoots were placed in some acetone and cut into small pieces. After extraction for half an hour at 20°C the solution was poured off, and the plant material was extracted for a further two hours and a half in ether, freed from peroxides. The acetone was evaporated off at 20°C and reduced pressure, whereupon the ether extract was added. In some cases the ether extract thus obtained was evaporated and the dry residue extracted with chloroform for 12 hours at 5°C. In other cases 25 ml. of distilled water were added to the ether extract, and the ether evaporated off at 30°C. The water solution thus obtained was filtered, and shaken first with ether at pH 8 (sodium bicarbonate), then with ether at pH 3 (tartaric acid). The alkaline ether fraction was discarded. The ether was evaporated from the acid ether fraction and the dry residue extracted with chloroform for 12 hours at 5°C.

From the chloroform solution the solvent was evaporated, and the dry residue was transferred, by means of ether, to paper chromatogram strips. The strips were 2.0 cm. wide, and the chromatograms were normally run until the front had ascended to a height of 15 cm. The chromatogram paper used was Whatman No. 1; the partition solvent was in some cases isopropanol/ammonia/water (10 : 1 : 1), in others ethanol/water (7 : 3). The further management of the chromatographic procedure was the same as is earlier described (Fransson 1958).

The chromatogram strips were sectioned and bioassayed partly in the wheat root cell elongation test, and partly in the *Avena* coleoptile section test. These two tests are used routinely at this institute and are earlier described (Burström 1942, Lexander 1953, Fransson 1958).

Chromatography with the Partition Solvent iso-Propanol/ Ammonia/Water

Strips with pine extract applied were run in isopropanol/ammonia/water (10 : 1 : 1) as partition solvent. In bioassay by means of the *Avena* coleoptile section test the different parts of the strips showed activities which are

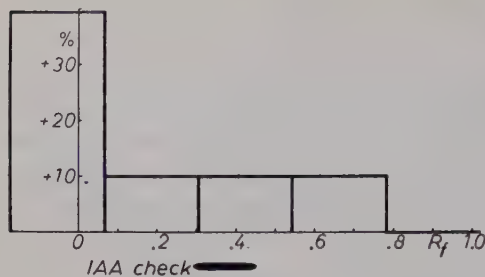


Fig. 1.

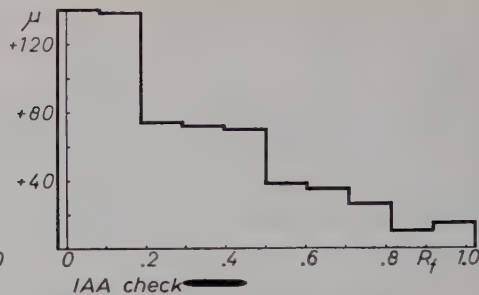


Fig. 2.

Figure 1. The activities of the acid ether fraction of pine seedling shoot extract in the *Avena coleoptile* section test. The fraction was chromatographed in isopropanol/ammonia/water (10 : 1 : 1). The activity is expressed as increase or decrease in respect to control section elongation. Fresh weight of plant material extracted 1.048 grams. The position of synthetic indole-3-acetic acid on a sprayed check strip is indicated.

Figure 2. The activities of the acid ether fraction of pine seedling shoot extract in the wheat root cell elongation test. The fraction was chromatographed in isopropanol/ammonia/water (10 : 1 : 1). The elongation is expressed as difference in cell-length in respect to that of control cells. Each length-value is a mean from 240 cells. Fresh weight of plant material extracted 1.048 grams. The position of synthetic indole-3-acetic acid on a sprayed check strip is indicated.

demonstrated in Figure 1. It is seen from the diagram that the pine extract contains a substance with auxin activity.

When a pine extract strip is bioassayed for its elongation activity on the wheat root cells a somewhat surprising result is obtained (Figure 2). It is seen that the strip contains an area stimulating root cell elongation, which does not point to the nature of an auxin.

It should be noticed that the concentration of pine substance applied to the strips is the same in the two cases (Figure 1 and Figure 2), and therefore the two strips are quite equal to each other.

It should also be noticed from the two figures that the activity in both cases has its highest magnitude in the area around the starting-line of the chromatogram strips. This circumstance indicates that the pine substance or substances streak, and the partition solvent isopropanol/ammonia/water has consequently been regarded as *not appropriate for chromatography of the actual pine extract*.

Chromatography with the Partition Solvent Ethanol/Water

Strips with pine extract applied were run using 70 per cent ethanol as the partition solvent, which solvent permits the pine substance to move as a

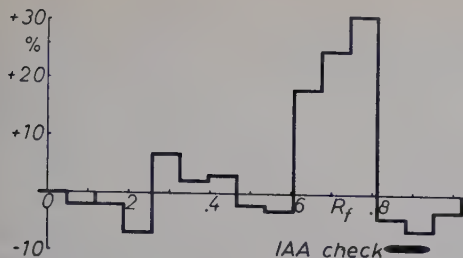


Fig. 3.

Figure 3. The activities of pine seedling shoot extract not treated with bicarbonate in the *Avena coleoptile* section test. The extract was chromatographed in 70 per cent ethanol. The activity is expressed as increase or decrease in respect to control section elongation. Fresh weight of plant material extracted 0.498 grams. The position of synthetic indole-3-acetic acid on a sprayed check strip is indicated.

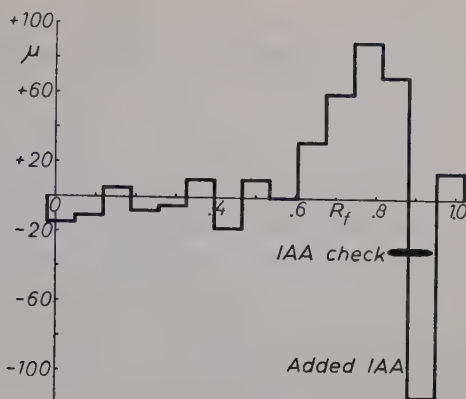


Fig. 4.

Figure 4. The activities of pine seedling shoot extract not treated with bicarbonate in the wheat root cell elongation test. Also pure indole-3-acetic acid was added at the starting-point of the chromatogram; its final concentration in the bioassay medium is about 10^{-7} M. The chromatogram was run in 70 per cent ethanol. The elongation is expressed as difference in cell-length in respect to that of control cells. Each length-value is a mean from 240 cells. Fresh weight of plant material extracted 0.498 grams. The position of synthetic indole-3-acetic acid on a sprayed check strip is indicated.

solute. This is evident from Figure 3, which shows the result from a bioassay of the strip in the *Avena* section test. It is seen from the figure that the active area has a R_f -value ($0.60 \rightarrow 0.81$) somewhat lower than that obtained for synthetic IAA on check strip ($0.83 \rightarrow 0.95$), which indicates that the two substances are not identical.

This is more conspicuous from Figure 4, which shows the result of the bioassay of the strip on root cell elongation. Before the running of the strip, also synthetic IAA was applied at the starting-point. The IAA was added in such an amount that its final concentration in the bioassay medium would be 10^{-7} M. As is seen in Figure 4, the main portion of the added IAA is found on the strip between the R_f -values 0.88 and 0.95 , while the pine substance has the values $0.60 \rightarrow 0.88$.

It should be noticed that equal amounts of pine extract were applied to both strips, represented in Figures 3 and 4.

It is evident from the two figures that IAA and the pine substance defini-

tively have different physiological properties; *the latter substance being a stimulator on both coleoptile and root cell elongation.*

Discussion

Considering the fact that the studied pine extract acts, at one and the same concentration, as a stimulator on the elongation of both oat coleoptile sections and root cells, it is obvious that the *pine substance is not identical with any of the now conventional native auxins*: indole-3-acetic acid, indole-3-acetaldehyde, indole-3-acetonitrile, methyl and ethyl indole-3-acetate or indole-3-pyruvic acid.

A suggestion of the existence of an auxin in pine not identical with IAA was already made in an earlier paper (Fransson 1953); that substance was there termed "pine auxin". Irrespective of whether the substances of that study and of this current one are identical or not, it is evident from the present results that the term "auxin" is not a correct one for the pine substance studied in this actual investigation. For the present it is therefore suggested that the substance be termed a pine growth substance *Pinus 1*.

In addition to the naturally-occurring auxins named above there is a group of native substances, not identified, which have auxin or auxin-like properties; these substances have recently been reviewed by Bentley (1958). In this group there are two substances, which on account of their physiological properties may be discussed together with the present pine growth substance. One of them is termed *accelerator- α* (Bennet-Clark and Kefford 1953), and the other is *compound W* (Housley and Bentley 1956).

Bennet-Clark and Kefford extracted growth substances from, *i.a.*, broad bean shoots and roots, and chromatographed the ether fraction containing the acid growth substances. These authors used *isopropanol/ammonia/water* as partition solvent. The chromatograms, when bioassayed, were found to contain a substance, which has a stimulating effect on the growth of both *Avena* coleoptile sections and sections of pea radicles. The R_f -value of this substance was decidedly lower than that of IAA; the authors suggested for the substance the term *accelerator- α* .

The substance *accelerator- α* has thereafter been reported to exist in a variety of plant tissues, *e.g.*, in wheat roots, bioassay: oat sections and root cell elongation (Lexander 1953); in apple leaves, bioassay: *Avena* coleoptile sections (Hancock and Barlow 1953); in broad bean shoots and roots, pea shoots and roots, sunflower shoots, maize roots, immature maize kernels, and potato shoots and tubers, bioassay: *Avena* coleoptile sections and pea root sections (Kefford 1955 a); in sunflower shoots, bioassay: pea root sec-

tions (Audus and Thresh 1956); further possibly in sweet corn seed, bioassay: *Avena* coleoptile sections and slit pea stems (Stowe and Thimann 1953); and possibly in pea roots, bioassay: *Avena* mesocotyl, *Avena* coleoptile and wheat coleoptile sections (Audus and Gunning 1958). In all these investigations reviewed the results were obtained by the bioassay of chromatograms.

The earlier investigations, by Lexander (1953) and by Kefford (1955 a), confirmed the observation of Bennet-Clark and Kefford that *accelerator- α* has a stimulating effect, even if it sometimes is weak, on the growth of shoots as well as of roots. Lexander showed furthermore that the growth of the roots is due to cell elongation.

This property of the substance seems, however, then to have been overlooked by some other authors, since they have in the first place studied the effect on the elongation of shoots. Instead the R_f -value has been taken as a weighty criterion of the existence of *accelerator- α* .

Bennet-Clark and Kefford obtained *accelerator- α* at a very low R_f -value; in reality their results show that the activity of the substance is highest at the starting-line of the chromatograms. In similar investigations other authors seem to have used too exclusively such partition solvents that on the whole give about the same R_f -values as those originally reported (Bennet-Clark and Kefford 1953). Such partition solvents are in the first place: *isopropanol/ammonia/water*, *isopropanol/water* (equilibrated), *n-butanol/ammonia/water*, *n-butanol/water* (equilibrated), and *isobutanol/methanol/water* (equilibrated). — For further information on the use of different partition solvents in paper chromatography, see, *i.a.*, the papers by Sen and Leopold (1954), Nitsch (1956), and Nitsch and Nitsch (1955).

In the investigations mentioned above *accelerator- α* is in most cases reported to be situated on the chromatograms at markedly low R_f -values, namely between 0.0 and approximately 0.2. It is at the same time striking that within this area the growth activity generally has its highest value at the starting-line. Considering these circumstances, it seems rather difficult to the present author to decide when only one type of solvent is used whether or not the found activity arises from one growth substance, or several growth substances, or from the total effect of growth substances and impurities. A R_f -value of a substance given as $0 \rightarrow 0.2$ with a peak activity at $R_f=0$, *i.e.*, on the starting-line, may not be the best criterion of the identity of the compound in question.

An important contribution to the knowledge about *accelerator- α* is given in a study by Housley and Bentley (1956) on extracts from the inner blanched leaves of cabbage. These authors found in the neutral ether fraction when chromatographed in *isopropanol/ammonia/water* a growth promoter, their compound *W*, situated between the R_f -values $0 \rightarrow 0.1$. This promoter was

shown to stimulate the growth of cress roots. However, the authors demonstrated that the *compound W* could be formed from a precursor present in the aqueous fraction by heat treatment followed by shaking with sodium bicarbonate solution. Such treatment is a common one in purification procedures of crude extracts. On account of the conformities in growth effects and R_f -values, Housley and Bentley suggest that *accelerator-a* and *compound W* may be one and the same substance. The authors also discuss at length the view that *accelerator-a* very likely belongs more to the neutral ether fraction, together with *compound W*, than to the acid fraction.

From the preceding it is thus obvious that there is a question whether *accelerator-a* and *compound W* are naturally-occurring substances or artefacts. In this connection it should be noticed that there are a number of studies of plant extracts where there is no trace of *accelerator-a* present in the acid ether fraction, see, e.g., Luckwill (1952), Fischer (1954), and Housley and Bentley (1956). It should also be emphasized that there are examples of investigations (Britton, Housley, and Bentley 1956), where the presence of *accelerator-a* sometimes can be demonstrated with success, but not always.

These circumstances, however, need not imply the non-existence of such natural growth substances that stimulate the growth (*i.e.*, the cell elongation) in both shoots and roots. That some substances can be thought to have such physiological properties is suggested by the results of Jerchel and Staab-Müller (1954) in a study on synthetic indole acetyl peptides. Some of these compounds are stimulators in the slit pea stem test, but they have hardly any auxin effects at all in cress root inhibition. Furthermore, it is reported by Fawcett, Wain, and Wightman (1955), and by Hansen, Burström, and Teär (1955) that synthetic indole-3-isobutyric acid has auxin activity in the *Avena* coleoptile section test, but also shows strong anti-auxin activity by stimulating cell elongation in wheat roots.

If it is assumed that *Pinus 1* has the same specific auxin activity as IAA this would mean that the activity in the pine shoots corresponds to an amount of about 98 µg. IAA per kilogram fresh material. The character of the pine substance of stimulating elongation in both shoots and roots can at these low amounts therefore not be explained by effects arising from nutrients.

The pine growth hormone *Pinus 1* studied in the present investigation thus shows some consistency with *accelerator-a* in regard to: (1) the position after chromatography in the solvent isopropanol/ammonia/water, and (2) the activity in both the *Avena* coleoptile section and the root cell elongation tests. That fact that the R_f -value is very low, so low that the highest activity after chromatography remains on the starting-line, is regarded as an indication

that the substance streaks. The partition solvent used must therefore be considered as an unsuitable one.

On the contrary, the partition solvent 70 per cent ethanol may be used with advantage. In this case *Pinus 1* is obtained within a rather compact area, having a somewhat lower R_f -value than that obtained with IAA.

After chromatography with 70 per cent ethanol as partition solvent *Pinus 1* retains its property of stimulating elongation in both shoots and roots. Here it differs markedly from the properties of IAA and related indole compounds, a fact that is well demonstrated from the results in Figures 3 and 4.

The discussion given above concerning the question of whether *accelerator-a* and *compound W* are naturally-occurring substances or artefacts (as a result from heat and sodium bicarbonate treatment) can of course to some degree also be applied to *Pinus 1*.

Judging from the results, however, it seems obvious to the author that the substance *Pinus 1* is not an artefact: 1. The typical nature of the growth stimulation remains, whether or not the substance has been treated with sodium bicarbonate solution (see, e.g., Housley and Bentley 1956); 2. This stimulation also remains, whether or not the chromatograms are run in ammoniacal medium (destruction in the presence of ammonia has been discussed by Nitsch 1956, and by Bentley *et al.* 1956). Therefore there seem to be rather good reasons for the suggestion that the substance *Pinus 1* is a naturally-occurring shoot and root cell elongation stimulator.

From the present results it cannot be decisively decided whether *Pinus 1* consists of more than one compound. This, however, does not seem very possible, as the compounds in such a case must have the same R_f -values and further, as the activity is found within a rather small area on the chromatograms; cf. the different R_f -values of the so-called interconvertible auxins: obtained from maize kernels (Avery and Berger 1943, Hemberg 1958), from tomato roots (Britton, Housley, and Bentley 1956), from pea roots (Audus and Gunning 1958), and possibly from cabbage (Prochazka, Sanda, and Sorm 1957).

A further support for the suggestion that *Pinus 1* is a natural growth substance is the fact that this substance is solely responsible for the whole growth activity on the chromatograms. There are no other peaks of growth activity, indicating stimulation or inhibition. It is evident from the other investigations reviewed in this paper that the unknown substances examined most frequently occur together with at least one of the conventional auxins, chiefly indole-3-acetic acid or indole-3-acetonitrile.

In the chromatograms a minimum amount of IAA of about 0.7 μ g. per litre can be determined with the test method used (cf. also Rietsema 1950). This amount should correspond to a concentration of 3.6 μ g. of free IAA

per kilogram of the fresh pine tissue. This is the upper limit of free IAA content that can be present in the extracts from the pine seedlings. This limit is, however, not reached in the pine extracts, whereas about 8—49 μ g. IAA per kg. fresh weight is found in, *e.g.*, broad bean shoots (Kefford 1955 b), in sunflower shoots, and in cabbage leaves (Audus and Thresh 1956). These circumstances therefore bring to the point that IAA probably can not be present in the pine extracts, or in the pine seedling shoots.

Summary

A growth substance is obtained in the acid ether fraction of extracts of pine seedling shoots. It is not examined whether the substance also occurs in the neutral ether or in the water fractions.

The substance is chromatographed, and bioassayed in the *Avena* coleoptile section and in the wheat root cell elongation tests.

It is found that:

1. The substance cannot be chromatographed with advantage in the partition solvent *isopropanol/ammonia/water*,
2. The solvent 70 per cent ethanol is applicable in chromatography; the pine substance shows a R_f -value of 0.74, while that of indole-3-acetic acid is 0.89,
3. The pine substance cannot be considered as an artefact but as a natural growth substance, which stimulates cell elongation in both coleoptiles and roots.

The author is very much indebted to Professor H. Burström for valuable criticism of the discussion of this study.

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On the Effect of Some Sugars and of 2,4-Dinitrophenol upon the Absorption of Phosphate Ions by Excised Roots

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Introduction

In previous communications the toxic effects of some sugars upon the accumulation of chloride and nitrate ions in roots was studied (Stenlid 1954, 1957 a, b). It was concluded that chloride and nitrate accumulation have different requirements of carbohydrates and that the absorption of the two ions is not affected in the same way by an addition of sugars. This is not surprising as it is known that nitrate accumulation is correlated with the assimilation of the ion. Nitrate assimilation in its turn is dependent upon a good supply of acceptors, which are probably derived more or less directly from sugars.

The uptake of phosphate ions is another example of ion absorption connected with sugar metabolism. In fact, it has been suggested that phosphate ions are absorbed combined with a sugar moiety (see *e.g.* Helder 1952, Lundegårdh 1955). Also in discussions of sugar absorption it has been surmised that the active accumulation occurring is coupled to the formation of hexose phosphates at the root surface. The opinions about the mechanism of sugar absorption in roots are still very different and in many respects unsettled (see discussions in Brown 1952, Rothstein 1954, Street 1957) but it seems plausible to assume that phosphate is involved at least in some cases. In view of this probably close relationship between sugar and phosphate uptake it seemed pertinent to investigate the effect of different sugars

upon phosphate uptake in excised roots, especially as the toxic effects exerted by certain sugars can be supposed to have some relation to phosphate metabolism.

Methods

The methods were mainly the same as in the experiments on chloride and nitrate uptake (Stenlid 1954, 1957 a, b). Excised roots of wheat seedlings prepared as previously (Stenlid 1957 b) were used. Cucumber roots (*Cucumis sativus*, Weibulls Västeråsgrurka) were taken from seedlings grown in the dark on moist filter papers in Petri dishes at 23°C. 3—4 days old roots with a length of 50—60 mm. were used. In some experiments excised roots from wheat plants about 14 days old were used. The plants were grown with artificial light for 12—14 days in nutrient solution (for the composition see Ekdahl 1953) and one day in distilled water. The roots (about 12—15 cm. long) were then excised and used in the absorption experiments.

The experiments were performed in flasks or large test tubes at 25°C and the solutions were aerated throughout the experiments. Duplicate samples of 2 ml. were removed from each tube for analysis of inorganic phosphate at the start of the experiment and after 4 hours. The phosphate uptake was calculated from the difference between the samples. The method of Fiske and Subbarow (1925) as modified by Umbreit *et al.* (1949) was used for the phosphate analyses. A Beckman photometer model DU was employed for the colorimetric determinations. The determinations were not significantly disturbed by sugars in the concentrations used in this investigation. Moreover, as the samples taken from one solution at 0 and 240 minutes were always analysed immediately after each other (and measured in the same cuvette) a possible small effect of the sugars will probably be eliminated. Also the errors caused by the fact that the blue colour is not quite constant are reduced when the samples are analysed in this way.

Chloride and nitrate were determined as described earlier (Stenlid 1957 b).

To make the experiments closely comparable with the experiments on chloride absorption, preliminary experiments were made with solutions composed of KCl, CaCl₂, KH₂PO₄ and Na₂HPO₄ in the same concentrations as in the chloride experi-

Table 1. Net uptake of inorganic phosphate and of total phosphorus from nutrient solutions. Excised roots pretreated with aerated distilled water were used. The values give the absorption in $\mu\text{g-atoms P/4 hours}$ and 100 mg. dry matter.

Roots used	Nutrient solution					
	Control		Control + glucose $10^{-2} M$		Control + mannose $10^{-2} M$	
	Inorg. P	Total P	Inorg. P	Total P	Inorg. P	Total P
Wheat roots pretreated 48 hours	0.7	0.6	2.9	2.1	4.3	4.1
Wheat roots pretreated 72 hours	0.8	0.0	2.6	2.9	3.0	3.2

Table 2. *Absorption of phosphate from solutions with different pH.* The values are the mean of three different determinations. Excised wheat roots used without pretreatment.

Composition of solution			pH		Phosphate absorbed, μg -ions per 4 hours and 100 mg. dry matter
KH_2PO_4	Na_2HPO_4	MgSO_4	Initial	Final	
$2.5 \cdot 10^{-4} \text{ M}$	$2.5 \cdot 10^{-4} \text{ M}$	$6 \cdot 10^{-4} \text{ M}$	7.0	5.8	3.6
$5 \cdot 10^{-4} \text{ M}$	—	$6 \cdot 10^{-4} \text{ M}$	5.5	4.7	3.8

ments. The absorption from these solutions was slow and rather irregular, however, and sometimes even an exudation of phosphate occurred. In experiments with additions of MgSO_4 or MgCl_2 it was found that Mg in most cases had a distinct positive and "stabilizing" effect upon phosphate absorption as compared to the corresponding calcium salts. The following solution was therefore used as a control unless otherwise stated: KH_2PO_4 $2.5 \cdot 10^{-4} \text{ M}$, Na_2HPO_4 $2.5 \cdot 10^{-4} \text{ M}$, MgSO_4 $6 \cdot 10^{-4} \text{ M}$, (pH about 6.8—6.9). The addition of further ions was avoided in order to cause as few complications as possible of the ion absorption, but preliminary experiments with other solutions seem to give about the same sugar effects as in the control solution described above. The absorption of sulphate ions or of the cations has not been determined.

It could be objected to this method that not the absorption of phosphate but the change in concentration of inorganic phosphate in the surrounding solution is measured. The decrease in phosphate content found might be due not to real absorption but to formation in the solution of phosphate esters which are not included in the analysis. This possibility was checked by analysing both total and inorganic phosphorus in some solutions (see Table 1). The analyses of total phosphorus were performed at the Department of Analytical Chemistry through the courtesy of dr. G. Köhler. The differences between the values for inorganic and total phosphate were found to be insignificant and a formation of organic phosphate in the solution cannot explain the decrease in content of inorganic phosphate. It may thus be concluded that the change in phosphate content is really due to absorption into the root.

In the present investigation the absorption was only slightly increased if the initial pH was changed from 7.0 to 5.5 (see Table 2, cf. Butler 1953 b). The experiments were performed in solutions with an initial pH of 6.8—7.0 (equal parts of primary and secondary phosphate) *i.e.* the same pH as used in the experiments with nitrate and chloride ions (Stenlid 1957 b). The pH drift during the experiments was somewhat more pronounced in the present investigation as root weight per volume was kept rather high to compensate for the slow absorption rate of phosphate (about 50—70 mg. dry matter/25 ml. were used). The total concentration of phosphate was lowered 20 per cent at most during the experiments.

The absolute amount absorbed by the controls varied somewhat more than in the chloride experiments. The effects of sugars were rather constant, however, and deviations exceeding 10 per cent of the control are to be regarded as significant.

The following abbreviations are used: 2-DG (2-desoxy-D-glucose), 2,4-D (2,4-dichlorophenoxyacetic acid), IAA (indole acetic acid), DNP (2,4-dinitrophenol), ADP (adenosinediphosphate), and ATP (adenosinetriphosphate).

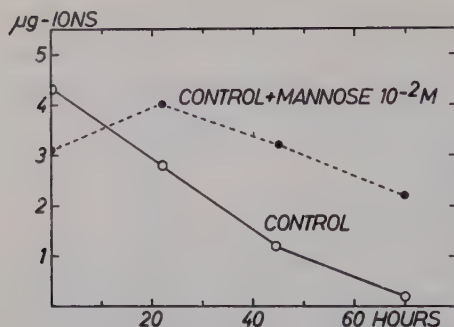


Figure 1. Absorption of phosphate from control solutions with and without mannose. All available data have been used for the curves and every point is the mean from at least 9 different tubes. On the abscissa time of pretreatment in distilled water, on the ordinate $\mu\text{g-ions}/4$ hours and 100 mg dry matter.

Effect of Sugars upon Phosphate Absorption

The absorption of phosphate ions by excised wheat roots from the control solutions without sugar is rather slow and proceeds at a much lower rate than the absorption of chloride and nitrate ions (Figure 1, cf. Stenlid 1957 b). Pretreatment of the excised roots with aerated distilled water at 20°C prior to the experiments results in a decreased uptake of phosphate ions. In this respect phosphate ions are similar to nitrate ions but differ from chloride ions. The uptake is calculated on a dry matter basis and as the quotient dry matter/volume decreases during the pretreatment a calculation per fresh matter or per volume will give a still stronger decrease. The decline in absorption capacity is more rapid for phosphate ions than for nitrate (cf. Stenlid 1957 b), and after 72 hours in most cases no phosphate at all is taken up, and an exudation of phosphate is frequently observed.

Turning now to the effects of sugars (Table 3), it is seen that *glucose* gives different effects upon phosphate absorption in non-treated and in pretreated roots. Small effects were found in freshly excised roots, and positive effects were obtained in pretreated roots. The results are of the same type as in the experiments on chloride and nitrate absorption. The uptake in pretreated roots from control+glucose was always less than from the non-treated roots and it does not seem possible to restore the full absorption capacity by adding glucose.

Galactose gives positive effects upon phosphate absorption in wheat roots irrespective of the pretreatment and it should be noticed that galactose has a more pronounced promoting effect than glucose. On the other hand, the positive effects upon respiration, chloride absorption and nitrate absorption are weaker for galactose than for glucose (see Stenlid 1957 b, 1959).

Mannose gives qualitatively different effects upon freshly excised roots and roots pretreated in aerated water. In roots which are not pretreated mannose inhibits phosphate uptake. The inhibition is much weaker than the inhibition of nitrate and chloride absorption (see Tables 3—4, cf. Stenlid

Table 3. *Effect of different sugars upon phosphate absorption in excised wheat roots. Relative values expressed as per cent of the absorption in control solution without sugar. The values are means of determinations from at least 6 different tubes.*

Pretreatment with aerated distilled water, hours	Absorption $\mu\text{g-ions/4}$ hours and 100 mg dry matter, no sugar added	Absorption, relative values with sugar added, M				
		0	Glucose 10^{-2}	Galactose 10^{-2}	Mannose 10^{-2}	2-DG 10^{-2}
0	4.4	100	99	115	72	57
20—24	2.5	100	105	154	144	—
44—48	1.6	100	143	233	259	135

1957 a, 1959). In pretreated roots the effect of mannose is changed and the absorption is greater if mannose is added. Like galactose, mannose gives even more conspicuous positive effects than glucose. The absolute amount of phosphate taken up in the presence of mannose may be greater in pretreated roots than in non-treated ones (Figure 1). This can be explained by assuming that more sites are available for the absorption in pretreated roots. Chloride and nitrate absorption is inhibited by mannose even in pretreated roots (Table 5, cf. Stenlid 1959). In Tables 5 and 6 simultaneous determinations of phosphate and chloride absorption from the same solutions are reported. It is seen that mannose has opposite effects upon the uptake of the two ions. The effects upon respiration, on the other hand, are similar to those upon phosphate absorption. In freshly excised roots mannose inhibits oxygen uptake, but in roots aerated for 40—50 hours positive effects are observed (Stenlid 1954, 1959). The cause of this reversed reaction of oxygen consumption to added mannose has not been investigated in detail, and it is not known if there is an increased consumption of mannose or if the effects are indirect. The agreement between oxygen and phosphate uptake is not absolute, and probably there is no quantitative relationship between the results. In roots aerated for 20 hours the respiration is, *e.g.*, somewhat inhibited by mannose but phosphate uptake is enhanced. Glucose gives a more vigorous stimulation of respiration than mannose and this also speaks against a direct quantitative correlation between the effects of sugars upon respiration and phosphate absorption.

Table 4. *Effect of mannose upon the absorption of phosphate by freshly excised wheat roots. Absorption expressed as $\mu\text{g-ions/4}$ hours and 100 mg. dry matter. The values are means of determinations from at least 6 different tubes.*

Conc. of mannose added to the control	0	10^{-3} M	$3 \cdot 10^{-3}$ M	10^{-2} M	$3 \cdot 10^{-2}$ M
Phosphate absorbed	4.3	3.8	3.5	3.0	2.2

Table 5. *Comparison of the effect of mannose upon the absorption of chloride and phosphate ions by excised wheat and cucumber roots.* Control solution: $\text{KCl } 5 \cdot 10^{-4} \text{ M}$, $\text{MgCl}_2 \text{ } 2.5 \cdot 10^{-4} \text{ M}$, $\text{KH}_2\text{PO}_4 \text{ } 2.5 \cdot 10^{-4} \text{ M}$, $\text{Na}_2\text{HPO}_4 \text{ } 2.5 \cdot 10^{-4} \text{ M}$. The values are means of samples from 3 different tubes.

Experiment	Roots	Pretreatment in aerated water	Solution	Absorption $\mu\text{g-ions/4 hours}$ and 100 mg. dry matter	
				Chloride	Phosphate
A	Wheat	20 hours	Control	27.8	3.4
A	Wheat	20 hours	Control + mannose $3 \cdot 10^{-3} \text{ M}$	13.1	5.2
B	Wheat	—	Control	12.7	5.0
B	Wheat	—	Control + mannose 10^{-3} M	4.8	4.8
C	Cucumber	20 hours	Control	40.5	3.2
C	Cucumber	20 hours	Control + mannose 10^{-2} M	20.7	4.7

The results with 2-desoxy-D-glucose (only a few experiments have been made, see Tables 3 and 6) are similar to those obtained with mannose.

In wheat roots mannose thus behaves towards the absorption of phosphate as galactose towards nitrate uptake (Stenlid 1957 b), being inhibitory in non-starved roots, but stimulating uptake in roots pretreated with aerated water. There is one important difference, however. Galactose was always found less efficient than glucose for nitrate uptake, but in pretreated roots mannose is more efficient than glucose for the absorption of phosphate. It can be assumed that galactose is utilized in some way for nitrate absorption, but that it is not as efficient as glucose. In roots with a relatively high content of glucose, galactose will be antagonistic to glucose but in starved roots with a low supply of glucose the competition is less important and galactose increases nitrate absorption. A similar hypothesis can scarcely explain the effect of mannose upon phosphate absorption without postulating some other difference between the two types of roots. If mannose is less efficient than glucose in non-treated roots it ought to be so in pretreated roots also, if the same mechanism is working. An explanation of the relatively weak effect of glucose in pretreated roots is that the competition for the added glucose from other reactions is strong in pretreated roots (where the concentration of endogenous sugar is low) so that the real concentration of glucose available for phosphate absorption will be low. Further experimental data including determinations of the rate of sugar uptake in pretreated roots are necessary for a definite explanation of the mannose effects.

The young roots used in this investigation may be thought to have another type of mechanism for phosphate uptake than roots from older plants where the grain is not an equally important source of mineral nutrients. Some experiments were therefore performed with wheat plants about 15 days old.

Table 6. *Effect of different sugars upon the absorption of phosphate and chloride ions in excised roots from wheat plants, 12—15 days old.* The excised roots were pretreated with aerated distilled water for 20 hours. Control solution as in Table 5. The absorption is given as $\mu\text{g-ions/4 hours}$ and 100 mg. dry matter.

Experiment	Ion determined	Addition to the control solution					
		0	Glucose $10^{-2} M$	Galactose $10^{-2} M$	Mannose $10^{-2} M$	Sucrose $10^{-2} M$	2-DG $10^{-2} M$
A	Chloride	9.2	16.7	—	1.5	14.6	—
A	Phosphate	— 0.5	1.7	—	2.0	1.9	—
B	Chloride	14.4	19.6	—	—	—	3.2
B	Phosphate	— 1.3	2.6	—	—	—	1.5
C	Chloride	12.3	16.7	13.1	—	18.9	—
C	Phosphate	0.7	2.2	2.1	—	2.0	—

Phosphate and chloride absorption was determined in excised roots in the same way as in the young roots (the composition of the nutrient solution was not the same, however). The effect of sugars was qualitatively the same in the old roots although exudation of phosphate often occurred in the control solutions (see Table 6). It seems therefore justified to suppose that there is no fundamental difference between the two types of roots but that in both types chloride and phosphate absorption differ in their reaction to sugars.

Cucumber roots behave somewhat different from wheat roots as the capacity of absorbing phosphate ions seems to be increased by pretreatment in aerated distilled water (Table 7). This suggests that the decreasing sugar content is less important than an increase in the absorbing capacity probably of the same type as found in the chloride experiments (cf. Stenlid 1957 b). Also in cucumber roots phosphate absorption is different from the absorption of chloride and nitrate. Under the experimental conditions mannose increases phosphate absorption whereas chloride and nitrate absorption is inhibited by mannose (Tables 5 and 7, cf. Stenlid 1959).

Table 7. *Effect of mannose upon phosphate uptake in cucumber roots.* Every value is the mean from at least six different tubes. The values give the absorption in $\mu\text{gions/4 hours}$ and 100 mg. dry matter.

Pretreatment of roots in distilled water, hours	Solution used	
	Control	Control + mannose $10^{-2} M$
—	2.7	2.9
22—24	4.2	4.8
42—48	4.0	—
68—72	3.1	4.6

Experiments with 2,4-Dinitrophenol

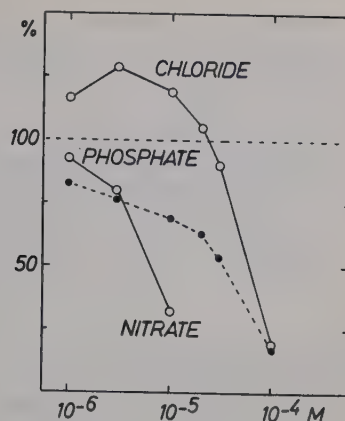
Inhibitors of oxidative phosphorylation such as methylene blue and 2,4-dinitrophenol (DNP) are known to inhibit the uptake of ions in plants (Stenlid 1950, Robertson *et al.* 1951, Lundegårdh 1952, Butler 1953 a, b, Ordin and Jacobson 1955). Particularly the phosphate uptake was studied by Mitsui and Kumazawa (1955), who found that DNP decidedly inhibited phosphate uptake in barley roots, and they point out that this is to be expected if phosphate accumulation is primarily and directly linked with the formation of energy rich phosphate in oxidative phosphorylation. The effect of DNP upon phosphate absorption in barley roots was investigated also by Hopkins (1956), who found that the absorption of both HPO_4^{--} and H_2PO_4^- was competitively inhibited by DNP, and by Hagen *et al.* (1957) who concluded that the phosphate uptake probably took place at sites identical with those for oxidative phosphorylation in mammalian mitochondria.

It is not clear how the oxidative phosphorylation is coupled to the ion uptake in general and probably several different effects of DNP (direct and indirect) must be assumed (see Robertson *et al.* 1955, Sutcliffe and Hackett 1957 and Lundegårdh 1958 b). In this connection it seemed pertinent to compare the effects of DNP upon the absorption of different ions. Some experiments were therefore made where the absorption of chloride and phosphate was determined simultaneously in the same solutions (see Table 8 and Figure 2). In concentrations between 10^{-6} M and 10^{-5} M DNP gave positive effects upon chloride absorption, whereas phosphate uptake was distinctly decreased. This suggests that different mechanisms are operative in phosphate and chloride uptake and that phosphate uptake is more directly

Table 8. *The effect of 2,4-dinitrophenol upon the absorption of chloride and phosphate ions by freshly excised wheat roots.* Control solution: KCl $9 \cdot 10^{-4}$ M, MgCl_2 $5 \cdot 10^{-5}$ M, KH_2PO_4 $2.5 \cdot 10^{-4}$ M, Na_2HPO_4 $2.5 \cdot 10^{-4}$ M. Every value is the mean of determinations from 3 different tubes.

Experiment	Ion determined	Solution used in the experiment				
		Control	Control + DNP 10^{-5} M	Control + DNP $2 \cdot 10^{-5}$ M	Control + DNP $3 \cdot 10^{-5}$ M	Control + DNP 10^{-4} M
A	Chloride	11.4	—	12.0	—	—
A	Phosphate	3.0	—	1.9	—	—
B	Chloride	13.4	15.7	—	10.5	3.0
B	Phosphate	4.5	3.4	—	2.5	1.2
C	Chloride	11.0	15.0	—	11.1	1.7
C	Phosphate	3.5	2.4	—	1.9	0.3
B + C	pH, initial	7.0	7.0	—	7.0	7.0
B + C	pH, final	5.4	5.9	—	6.3	6.6

Figure 2. Effect of DNP upon the absorption of nitrate, chloride and phosphate in excised wheat roots. Every point is the mean of determinations from at least six (for $2 \cdot 10^{-5}$ M three) different tubes. On the abscissa concentration of DNP, on the ordinate absorption % of control.



correlated with oxidative phosphorylation. Nitrate uptake, which was determined in separate experiments was affected in about the same way as phosphate uptake (Table 9). The effects of DNP upon the absorption of the three ions are compared in Figure 2.

The concentrations of DNP which induce an increased chloride absorption also stimulate oxygen uptake (see Stenlid 1949). In higher concentrations of DNP the stimulation of oxygen uptake is more pronounced, but the chloride accumulation is strongly inhibited (cf. experiments with carrot tissue reported by Robertson *et al.* 1951 and with wheat roots by Lundegårdh 1952 and Butler 1953 a). The inhibition of chloride absorption may quite well be of another nature than the inhibition of phosphate uptake occurring at lower concentrations (perhaps indirect effects such as a gradual disorganization of the mitochondria contribute).

The stimulation of chloride accumulation at low concentrations of DNP is an interesting phenomenon. Almost all earlier investigations have shown an inhibition of ion absorption (in Lundegårdh's paper 1952 a tendency to

Table 9. Effect of DNP upon nitrate absorption in freshly excised wheat roots. Composition of control: KNO_3 $9 \cdot 10^{-4}$ M, $\text{Ca}(\text{NO}_3)_2$ $5 \cdot 10^{-5}$ M, KH_2PO_4 $2.5 \cdot 10^{-4}$ M, Na_2HPO_4 $2.5 \cdot 10^{-4}$ M. Every value is the mean from 6 different tubes.

Absorption of nitrate	Concentration of DNP added to the control			
	0	10^{-6} M	$3 \cdot 10^{-6}$ M	10^{-5} M
$\mu\text{g-ions/4 hours and 100 mg. dry matter}$	18.8	17.5	15.1	6.1
per cent of control	100	93	80	32
pH, initial	6.9	6.8	6.7	6.6
pH, final	5.6	5.7	5.7	5.9

Table 10. *Effect of salicylic acid upon the absorption of phosphate and chloride in freshly excised wheat roots.* The absorption was determined simultaneously in the same solutions. Control solution as in Table 5. Every value is the mean of determinations from 3 different tubes.

Absorption $\mu\text{g-ions/4 hours}$ and 100 mg. dry matter	Concentration of salicylic acid added to the control				
	0	$3 \cdot 10^{-6} \text{ M}$	10^{-5} M	$3 \cdot 10^{-5} \text{ M}$	10^{-4} M
Phosphate	3.3	3.2	3.1	2.7	1.9
Chloride	12.5	12.8	15.3	15.8	15.7

increased absorption may be traced in Figure 25, however, and Chasson and Levitt 1956 in a preliminary note report DNP to stimulate calcium uptake in potato tuber). It is not easy to explain the positive effect, but as regards chloride absorption it might be a result of the increased oxygen uptake *per se*, leading to an enhanced transport via the "cytochrome ladder" in the sense of Lundegårdh (1955). The effects of DNP are far from fully understood, however, and other tentative explanations are possible. *E.g.*, it can be mentioned that some preliminary experiments on the interaction between DNP and auxins suggest that DNP has some antiauxinic properties. DNP may namely reverse the inhibitory effects of 2,4-D and IAA upon wheat root elongation (see Table 11, cf. Pohl and Ochs 1953). The present results thus show that the effects of DNP resemble those of salicylic acid more than was supposed in a previous paper where these effects of DNP had not been observed (Stenlid 1957 b). Salicylic acid affects the absorption of nitrate, chloride and phosphate in about the same way as DNP (see Stenlid 1957 b and Table 10). There are still important differences, however, especially as regards the effects upon oxygen consumption, where salicylic acid does not give any positive effects comparable to those obtained with DNP.

Table 11. *Growth of wheat roots in nutrient solutions with addition of DNP in combination with IAA, 2,4-D, mannose, and galactose.* Values in per cent of the growth in control solution during 24 hours at 20°C (the growth of the control varied between 24 and 27 mm). pH about 6. Every value is the mean of at least 60 roots from two different experiments. For further details about the growth experiments see Stenlid 1957 a.

Addition to control solution	Concentration of DNP					
	0	10^{-7} M	$3 \cdot 10^{-7} \text{ M}$	10^{-6} M	$3 \cdot 10^{-6} \text{ M}$	10^{-5} M
—	100	102	105	102	100	67
IAA, $3 \cdot 10^{-8} \text{ M}$	40	—	—	70	72	57
2,4-D, $2 \cdot 10^{-7} \text{ M}$	59	—	64	84	87	—
Mannose, $5 \cdot 10^{-4} \text{ M}$	38	—	—	56	68	—
Galactose, $5 \cdot 10^{-4} \text{ M}$	54	—	—	70	72	—

Another pertinent result is that the mannose and galactose inhibitions of wheat root growth are partly reversed by DNP (see Table 11). This may be due to impeded transport of the toxic sugar (perhaps as a result of inhibited oxidative phosphorylation, cf. Stenlid 1949), and it can be mentioned that also the transport of auxins is inhibited by low concentrations of DNP (see Niedergang-Kamien and Leopold 1957). It is not possible to discuss the antagonistic effects of DNP against sugars and auxins in any detail at present, but the data show that other effects of DNP than the direct effect upon oxidative phosphorylation are of importance in plant roots.

Discussion

The absorption of phosphate is in this paper taken in a rather wide sense and is meant to include all the phosphate disappearing from the nutrient solution surrounding the root. No attempt has been made to distinguish between different types or phases of the absorption. There are several suggestions that the phosphate uptake has special features different from the uptake of other ions (see Helder 1952, Humphries 1952, Lundegårdh 1955, Robertson 1958). Robertson's suggestion: "Information on the mechanism of uptake of phosphate, which occupies a special place in the metabolism of cells, may have little relation to the mechanism of uptake of other ions" gives an accentuated expression of these views. Before the absorption is discussed in relation to sugar metabolism, a short summary of the characteristics distinguishing phosphate absorption from the absorption of other ions will be given.

1) In nutrient solutions phosphate is mainly present as the two ions HPO_4^{--} and H_2PO_4^- , the relative amounts of which are changed with pH. As a rule the absorption of phosphate increases at lower pH values in the range pH 5—7 (van den Honert 1933, Olsen 1953, Hagen and Hopkins 1955) and it seems probable that this is mainly due to easier absorption of the monovalent ion, which predominates at pH values below 6 (at pH 6 about 90 per cent is present as H_2PO_4^-). The absorption mechanism becomes saturated at low concentrations (van den Honert 1933, Hagen and Hopkins 1955) and already at the concentrations used in this investigation (0.5 mM) the influence of pH will be rather small below pH 7). H_2PO_4^- and HPO_4^{--} are probably absorbed via different sites in the roots (Hagen and Hopkins 1955).

2) The initial concentration in the roots is higher for phosphate than for chloride and the accumulatory mechanism in most cases has to work against a concentration gradient from the beginning.

3) The rate of uptake is rather slow for phosphate compared to nitrate and chloride. In many cases a loss of phosphate occurs from roots which absorb a considerable amount of chloride. The loss of phosphate is counteracted by addition of sugars (Michael and Marschner 1958, cf. Table 6).

4) Pretreatment with distilled water decreases the capacity for absorbing phosphate in wheat roots, but promotes chloride absorption.

5) It has been demonstrated that sugars may stimulate the uptake of phosphate more than that of other ions (see *e.g.* van Andel *et al.* 1950, Helder 1952). Mannose and 2-desoxy-D-glucose (2-DG) stimulate the absorption of phosphate but inhibit the absorption of chloride and nitrate in excised wheat roots pretreated with water.

6) Divalent ions, especially Mg^{++} , stimulate the uptake and transport of phosphate. Magnesium phosphates are particularly efficient as phosphorus fertilizers (see Jacob 1955 for a review) which has been explained by the assumption that magnesium phosphate is taken up more readily than other phosphates.

7) Phosphate absorption is more easily inhibited by additions of dinitrophenol than chloride absorption.

8) Inorganic phosphate absorbed from nutrient solutions is rapidly incorporated into organic compounds, especially ATP and ADP (Loughman and Russell 1957, Miettinen and Savioja 1958).

These last-mentioned investigations with labelled phosphate showing that external phosphate is transferred into organic esters are of a special interest for a discussion of the absorption mechanism. ATP and ADP seem to be the first intermediates both in pea and barley roots, and a metabolic steady state with respect to these nucleotides is reached within 5 minutes. The formation of labelled ATP was found to be inhibited by DNP (Loughman and Russell 1957). The labelled phosphate was also detected in *i.a.* glucose-6-phosphate, fructose-6-phosphate and fructose-1,6-diphosphate, but these compounds seem to be formed after ATP and ADP. In addition, other nucleotides than ADP and ATP are probably formed.

These results together with the results obtained by Mitsui and Kumazawa (1955), Hopkins (1956), Hagen *et al.* (1957), and the experiments reported in Table 8 and Figure 2 make it probable that the absorption of phosphate is at least partly coupled to the formation of ATP through oxidative phosphorylation.

The effect of sugars upon phosphate absorption is certainly not a simple one. Some sugars, *e.g.* glucose and galactose, increase the uptake also of other ions (chloride and nitrate) at least under certain conditions. If the supply of carbohydrates is suboptimal there is without doubt a general positive effect upon ion accumulation of the addition of sugars. This effect can be assumed for all ions, both metabolically inert, such as chloride, and ions which are rapidly metabolized, such as nitrate and phosphate.

For nitrate and phosphate special effects are obtained besides the general effect. The reduction products of nitrate ions require suitable acceptors, and the addition of sugars can give rise to a more rapid absorption via the production of these acceptors.

Phosphate ions behave similarly to nitrate ions in several respects. Pretreatment of wheat roots with distilled water results in a lower absorption

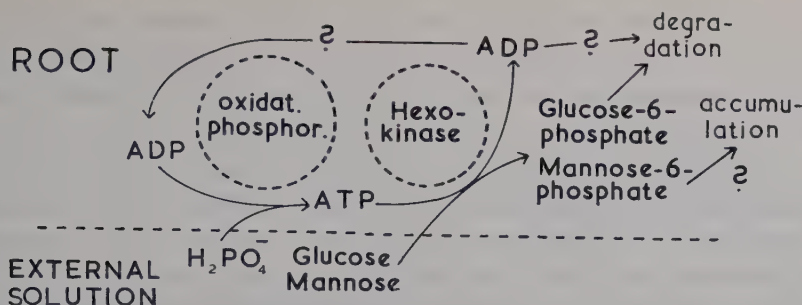
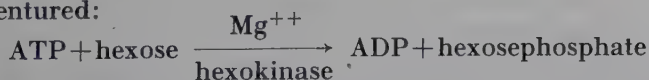


Figure 3. *Tentative scheme of the participation of hexoses in phosphate absorption.*

of both ions in contrast to what is found for chloride ions (see Stenlid 1957 b), and sugars give more distinct effects than in chloride experiments. Salicylic acid and 2,4-dinitrophenol inhibit the absorption of nitrate and phosphate when added in concentrations that stimulate the uptake of chloride. Of great interest is that nitrate and phosphate differ in their behaviour towards mannose and 2-DG in wheat roots. Nitrate absorption is always distinctly inhibited by mannose (Stenlid 1959) but phosphate absorption is enhanced in pretreated roots and only slightly retarded in freshly excised roots. 2-DG, which has only been tested in a few experiments, seems to have about the same effect as mannose. Galactose has somewhat different effects upon the two ions; nitrate uptake is retarded in non-pretreated roots, whereas phosphate absorption is increased in the same type of roots.

The effects of sugars upon phosphate absorption are thus probably not the same as upon nitrate absorption. The differences in the reaction to applied sugars can be explained *e.g.* by the assumption that phosphate reacts with the sugars themselves but that nitrate uptake is promoted by some degradation product which is not formed from mannose. It is difficult to decide the exact nature of the sugar effect in phosphate uptake, but as a tentative hypothesis the participation of a reaction of the hexokinase type may be ventured:



As mentioned above, ATP is probably formed in the initial phase of phosphate absorption and hexokinase seems to be rather common in plants (Saltman 1953). The positive effects of magnesium upon phosphate absorption is easily explainable if a reaction of the hexokinase type is operative, as the key role of magnesium in the enzyme systems necessary for the metabolism and phosphorylation of carbohydrates (*i.a.* hexokinase) is well established (for a review see Hewitt 1958). The assumptions about phosphate absorption

in roots after the addition of external sugars are combined into a hypothetical scheme in fig. 3, the net result of which is formation of sugar phosphate from phosphate ions and hexoses. A similar pattern for phosphate uptake has been suggested for yeast cells (see Rothstein 1954).

Referring to investigations on the occurrence of phosphorylations in plant roots Lundegårdh (1955) gives a hypothetical scheme in which inorganic phosphates are coupled to sugars at the root surface (under formation of hexose-phosphate) and released again in the interior of the root. He declares that it is an open question if such a mechanism really operates in the root. In an experimental test of the hypothesis with potato tissue Lundegårdh (1958 a) arrives at the conclusion that in potato tuber tissue the uptake of phosphate is rather similar to the uptake of chloride ions and that phosphorylations play no special role in the absorption of phosphate ions. It thus seems possible that the mechanism of uptake is different for potato tuber tissue and wheat roots.

From an analysis of the influence of pH upon phosphate absorption in barley roots Hagen and Hopkins (1955) concluded that the rate-limiting process involves the cleavage of a bond between an oxygen atom and an organic residue such as the glucosidic bond in glucose-phosphate. This conclusion is consistent with the hypothesis that the formation of ATP or a sugar phosphate is a part of the absorbing mechanism.

The positive effects of mannose and 2-DG are explicable by the hypothesis outlined in Figure 3, as both sugars are phosphorylated by hexokinases (see Sols *et al.* 1958). Goodman and Rothstein (1954) found that both glucose and mannose induced an uptake of phosphate in yeast. Galactose seems not to be a suitable substrate for ordinary hexokinases (see Dixon and Webb, 1958, Sols *et al.* 1958). The distinct positive effects of galactose upon phosphate absorption can therefore not be explained as due to the activity of hexokinase. One possible explanation is that galactose is transformed to glucose and that glucose is the real phosphate-consuming substance after the addition of galactose. As galactose in some types of roots causes a more pronounced consumption of phosphate than glucose, such a hypothesis is not probable, however. Another result speaking against a conversion of galactose to glucose in wheat roots is that galactose is toxic to wheat root growth and that this toxicity is counteracted by glucose (see Stenlid 1957 a). The existence of a special enzyme forming galactose-1-phosphate from ATP and galactose is another possible explanation of the positive galactose effects. Such galactokinases are rather common in different organisms, and although they have not been demonstrated in roots they may quite well be present (on the occurrence of a galactokinase in wheat germs see Albaum 1958). The

rapid absorption of galactose by wheat roots (Stenlid 1957 a) speaks in favour of some special enzyme catching this sugar.

The positive effects of mannose, galactose and 2-DG upon the respiration in starved wheat roots (Stenlid 1959) show that these sugars may become involved in oxidative metabolism, although it is not proved that the effects are really due to oxidation of the sugars. In any case it is probable that some phosphates are formed, and the toxicity may be due to these phosphates, especially if they are slowly metabolized and therefore accumulate in concentrations which may inhibit some enzyme. It has been suggested that the galactose toxicity, which is sometimes observed in animal tissues, is due to galactose-1-phosphate (Ginsburg and Neufeld 1957, Sidbury 1957). This phosphate inhibits phosphoglucomutase and the inhibition is overcome by glucose-1,6-diphosphate.

The available data about phosphate absorption in roots are too scanty to allow a detailed interpretation of the effects obtained after addition of different sugars. The multitude of reactions in the carbohydrate degradation which are coupled to production and consumption of ATP and ADP make it very difficult to predict how an addition of sugars may influence the reactions in the scheme outlined above, especially as little is known about how the substances are transferred from one reaction to another. ADP is *e.g.* formed in the hexokinase reaction, but is it possible that this ADP may be utilized in the oxidative phosphorylations? If it is, a limited cycle can be established transforming inorganic phosphate to hexose-6-phosphate via ATP. Another pertinent question is if all the ATP formed at various places (glycolysis, Krebs' cycle, oxidation of cytochromes etc.) is available for the hexokinase reaction.

In the presence of sugars, ADP should thus take part in the first and ATP in the second part of phosphate uptake, and it is not possible to predict how alterations in the quotient ADP/ATP should affect phosphate absorption. ADP is required in some rate-determining reactions in the degradation of sugars and these reactions may compete with the mechanism of oxidative phosphorylation for ADP. On the other hand, several reactions in the degradation of sugars include oxidative phosphorylation (which can increase phosphate uptake) and production of ATP (for a review see Krebs and Kornberg 1957). It might be possible that mannose by being metabolized at a low rate is accumulated as mannose-phosphate, thereby increasing the supply of ADP and the absorption of phosphate (provided that ADP is rate-limiting for the phosphate uptake). Hagen *et al.* (1957) found that externally applied ADP contrary to what is to be expected inhibited phosphate uptake. As one possible explanation they suggest that the location of ADP in the roots

is important and that externally applied ADP is not equivalent to the native ADP.

Different sugars have diverging effects also on other processes, *e.g.* growth, which certainly effects the ATP concentration in roots. Keeping all the possible complications in mind, it seems as if speculations about the details in the effects of sugars are of limited value without further data about the metabolism of different sugars in the root.

Summing up it can be said that sugars certainly stimulate phosphate absorption in a specific way, which is not identical with the effect of sugars upon chloride and nitrate absorption, and that coupling of sugars to phosphate is a possible reaction during phosphate absorption in roots. But is it also an important or even a normal pathway? The findings in investigations with labelled phosphate show that the phosphate seems to be incorporated into ATP prior to the formation of sugar phosphates. On the same time labelled inorganic phosphate was found in the root, and obviously it is impossible to decide which way the phosphate takes from a mere determination of the amounts of the different substances, especially as inorganic phosphate may be formed anew from the organic esters. Even if the coupling in oxidative phosphorylation is the first phase in an absorption mechanism, phosphate may be absorbed in other ways too, perhaps by an unspecific anion absorbing mechanism. Pathways other than those in excised roots might be followed in intact plants where a transport to the shoot takes place.

Summary

Phosphate absorption has been studied in excised roots from 3—4 days old seedlings of wheat and cucumber. The roots were used directly after excision or after pretreatment for 20—70 hours in aerated distilled water. In freshly excised wheat roots mannose and 2-desoxy-D-glucose (2-DG) had an inhibitory effect upon phosphate uptake which was less pronounced than upon chloride and nitrate absorption. Phosphate uptake in pretreated roots was stimulated by glucose, galactose, mannose and 2-DG. Chloride and nitrate uptake in pretreated roots is inhibited by mannose and 2-DG and stimulated by glucose and galactose.

2-4-Dinitrophenol (DNP) in concentrations between 10^{-6} M and 10^{-5} M inhibited phosphate and nitrate uptake but stimulated chloride uptake in freshly excised wheat roots. In higher concentrations DNP inhibited the uptake of all three ions.

It is concluded that the mechanism for phosphate uptake is different from the mechanism for chloride and nitrate uptake, and that oxidative

phosphorylation and coupling to sugars are of special importance for phosphate absorption.

Some growth experiments are reported showing that DNP may reduce the inhibitory effects of auxins, galactose and mannose.

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Species Differences between Plant Roots in the Reaction to Inhibitory Sugars

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Introduction

Some sugars are toxic to plants, probably through displacement of necessary sugars, and especially the effects of galactose and mannose have been studied (see Stenlid 1954, 1957 a, b, Street 1957, Ferguson *et al.* 1958 a, b). The toxic effects of these two sugars are interesting as both occur in different polysaccharides and glucosides in the plant kingdom.

It is not easy to obtain a clear-cut picture of the toxic effects with regard to different species. In most cases the effect upon growth and development has been studied. Much of the work has been performed with isolated roots or tissue cultures. The organ is then totally dependent upon external carbohydrates and the results are not fully comparable with results obtained with intact plants, especially as different methods and plants of different age have been used in different investigations. Some authors (*e.g.* Knudson 1917 and Farkas 1954) found galactose and mannose toxic to all species tested, and Farkas explicitly states galactose toxicity to be a universal phenomenon among higher plants. In other investigations reports of non-toxicity of the sugars are found, however. Wachtel (1943) found galactose non-toxic to *Cardamine* seedlings, and Reinholz (1954) got normal germination of cress in both mannose and galactose. Wynd (1933) even reported mannose to be a better carbon source for orchid seedlings than glucose and sucrose. Gautheret (1945) working with carrot root tissue found that both galactose and mannose supported the growth of the tissue. Burström (1948) observed no toxic

effects of galactose upon sunflower and flax roots, whereas Farkas (1954) and Ferguson *et al.* (1958 b) found galactose toxic to flax. As a whole the available data are too scanty to permit any conclusions about the universality of the toxic effects or the possible confinement of them to special systematic groups.

In stem and leaf tissues other conditions than in roots seem to prevail (see Ball 1955 and Stenlid 1957 a for references) and the present discussion will be confined to roots. In previous communications (Stenlid 1954, 1957 a, b) some processes known to be dependent upon a good carbohydrate supply were studied in wheat roots, namely growth, respiration, chloride absorption and nitrate absorption. In the present investigation the same processes are studied in roots from some other plants. It was planned to include several species in addition to those actually mentioned in this paper, and they were also tried in preliminary experiments. Of most species it was difficult to obtain sufficient and uniform material, however, or the data from the experiments varied too much to allow any definite conclusions. It has therefore not been possible to find any general difference between families or larger systematic units, but the results may anyhow be of interest as a contribution to the understanding of the physiology of sugars in plants.

Growth Experiments

The root growth of intact seedlings was measured in nutrient solutions with additions of different sugars. The methods were mainly the same as used previously for wheat roots (Stenlid 1957 a). The following species were tested: Cucumber (*Cucumis sativus*, Weibulls Västeråsgrurka), flax (Svalöfs Concurrent), cress (*Lepidium sativum*, Weibulls trädgårdskrasse nr 375), pea (Svalöfs Torsdags III), vetch (*Vicia sativa*, Svalöfs Stjärnvicker), clover (*Trifolium pratense*, Weibulls resistent), wheat (Svalöfs spring wheat Diamant II), oats (Svalöfs Solhavre), rye (Svalöfs Stålråg), barley (Svalöfs Ymerkorn), and maize (hybrid maize, Wisc. 464). The nutrient solution used by Ekdahl (1953) was used in most cases. In the experiments with flax and cucumber roots the solution used by Åberg (1950) was employed. The growth of the control and the effect of different sugars were about the same when the two solutions were tested with the same species. The solutions were saturated with air before the experiments were started and in most cases aeration during the experiments was without any clear effect. No further aeration was therefore applied. Only in the experiments with cress roots the solutions were aerated during the experiments, as this resulted in better growth of the control roots. The growth period was 24 hours at 20°C in the dark unless otherwise stated. For each solution three cork disks with seedlings were placed in beakers with 300 ml of the solution. The root length at the start of the experiments was as a rule 15–25 mm. (for flax roots 7–8 mm. and for cress and clover roots 12–15 mm.). 15 (of maize and pea 5) seedlings were placed at each cork disk. The 10 (of maize and pea 3) roots from each cork disk showing the best growth were used for the calculations.

Table 1. *The effect of mannose, 2-DG and galactose upon root growth in different species. The values give the growth in per cent of the control without sugar.*

Sugar added, M	Flax	Pea	Vetch	Clover	Maize	Oats	Rye	Barley
Mannose $3 \cdot 10^{-4}$	43	—	—	—	—	—	80	—
" 10^{-3}	18	108	—	—	—	54	24	54
" $3 \cdot 10^{-3}$	—	104	—	33	—	—	16	—
" 10^{-2}	—	64	—	—	—	—	—	—
2-DG 10^{-4}	37	91	—	—	77	—	—	—
" $3 \cdot 10^{-4}$	11	—	—	—	40	—	—	—
" 10^{-3}	—	36	—	29	—	—	—	—
Galactose 10^{-4}	—	—	—	—	—	—	—	83
" $3 \cdot 10^{-4}$	105	—	—	—	—	—	—	43
" 10^{-3}	95	—	—	—	86	24	31	16
" $3 \cdot 10^{-3}$	76	121	—	85	—	—	—	—
" 10^{-2}	52	109	91	—	65	—	—	—

All experiments were repeated at least once and the values are the mean of at least 6 cork discs. The growth per 24 hours of the controls without sugar was 23—27 mm. for wheat and maize roots, 18—21 mm. for cucumber, flax and pea roots, 20—23 mm. for vetch, barley, oats and rye, and 10—14 mm. for cress and clover roots.

Unless otherwise stated the D-forms of the sugars were used. 2-desoxy-D-glucose is abbreviated 2-DG.

The main results are summarized in Table 1 and Figures 1—2. It is seen that mannose and 2-DG are toxic to all species tested and that 2-DG always is the more toxic of the two sugars. Pea roots are less sensitive to the inhibitory sugars than the other species tested. This may be connected with the greater thickness of the pea roots compared to the other roots used, or it may be due to their higher sugar content (Table 5). As pea roots react differently in many respects, some fundamental deviation in the metabolism is possible, however.

An interesting result is that galactose seems to be non-toxic to some species. Cucumber and cress roots were studied more closely, and no inhibitory effects upon growth during 24 hours were obtained in galactose concentrations up to 0.01 M (Figures 1 and 2). Instead a stimulation was observed, especially upon cress roots. Reinholz (1954) also found that galactose (0.05—0.2 M) stimulated germination and root growth in cress. She reports mannose as inactive in the same investigation, and it is thus uncertain if the cress roots reacted in the same way in the two investigations.

In cress and cucumber roots the inhibition caused by mannose and 2-DG can be reversed through additions of galactose, glucose, and sucrose (Tables 2 to 4).

There are at least two different ways of explaining the non-toxicity of galactose to cucumber and cress roots, namely:

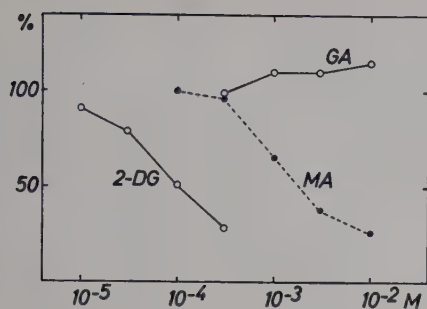


Fig. 1.

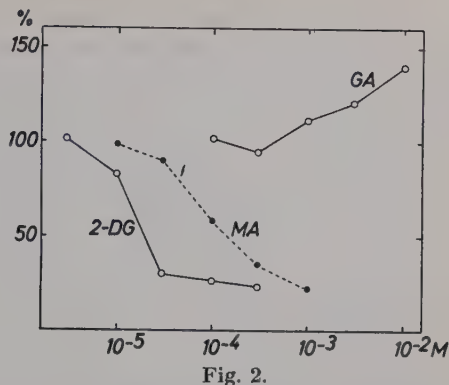


Fig. 2.

Figure 1. Growth of cucumber roots in nutrient solution with the addition of D-mannose (MA), D-galactose (GA), and 2-desoxy-D-glucose (2-DG). On the abscissa sugar concentration, on the ordinate growth as per cent of control.

Figure 2. Growth of cress roots in nutrient solution with the addition of mannose, galactose, and 2-DG. For further explanations see fig. 1.

- 1) The growth mechanism is not inhibited by galactose.
- 2) Galactose is converted to glucose so rapidly that it does not reach the growth mechanism in sufficiently high concentration to give toxic effects.

If galactose is non-toxic in itself, it seems as if it should be capable of reversing the toxic effects of mannose and 2-DG. Galactose may either be an indifferent antagonist only preventing the toxic sugars to exert their effect, or it may be positively utilized in the growth process.

Galactose is more effective than sucrose in reversing the growth inhibition caused by mannose and 2-DG in cucumber roots. In wheat roots sucrose is a rather effective antagonist against the inhibitory sugars (Stenlid 1957 a) although it gives toxic effects in the cultivation of excised roots (Burström 1941). Ferguson *et al.* (1958 b) in experiments with tomato roots found that

Table 2. Reversal of mannose inhibition of growth in cucumber roots by some other sugars. Concentration of mannose $3 \cdot 10^{-3}$ M. The values give the growth in per cent of control without sugars.

Concentration of reversing sugar	Reversing sugar added			
	Glucose	Galactose	Xylose	Sucrose
0	45	45	35	38
10^{-3} M	80	58	37	46
$3 \cdot 10^{-3}$ M	104	77	59	55
10^{-2} M	105	107	—	58

Table 3. *Reversal of the 2-DG inhibition of root growth in cucumber roots by some other sugars. The values give the growth in per cent of control without sugars.*

Concentration of reversing sugar	Reversing sugar added								
	Glucose		Galactose		Fructose	Xylose	Sucrose		L-Arabinose
	Concentration of 2-DG, M								
	10 ⁻⁴	3 · 10 ⁻⁴	10 ⁻⁴	3 · 10 ⁻⁴	10 ⁻⁴	10 ⁻⁴	10 ⁻⁴	3 · 10 ⁻⁴	10 ⁻⁴
0	58	22	55	22	43	43	53	33	43
3 · 10 ⁻⁴ M	72	30	74	—	—	59	62	—	—
10 ⁻³ M	86	49	93	45	—	59	67	—	—
3 · 10 ⁻³ M	88	68	102	65	63	62	70	—	47
10 ⁻² M	—	—	—	—	—	67	78	76	—

sucrose was a much less effective reversing agent than glucose towards mannose and galactose. In supporting growth, on the other hand, they found sucrose to be a far better carbohydrate source than glucose. The splitting of sucrose by plant roots and the possible complications during its uptake make an interpretation of the sucrose effects difficult.

A striking illustration of the difficulties met with in interpretations of the effects of mannose and galactose was found in experiments on wheat root growth, where both sugars were given simultaneously (see Table 6). The table is combined from experiments performed at different times and the variation is due to differences in the quantitative reaction of the roots in different seasons and years. There is, however, a general trend common to all the experiments. When given alone to the nutrient solutions the sugars produce negative effects. In the presence of mannose, however, galactose stimulates root growth, and so does mannose in the presence of galactose, provided the concentrations lie within a certain range. *E.g.*, it is seen in

Table 4. *Growth of cress roots as affected by mannose and 2-DG alone and in combination with other sugars. The values give the growth in per cent of control without sugar.*

Reversing sugar added, M	Inhibiting sugar added, M				
	Mannose		2-DG		
	3 · 10 ⁻⁴	3 · 10 ⁻³	3 · 10 ⁻⁵	10 ⁻⁴	3 · 10 ⁻⁴
0	43	25	27	22	19
Glucose 10 ⁻⁴	70	—	—	—	—
„ 3 · 10 ⁻⁴	82	—	—	34	—
„ 10 ⁻³	—	—	77	64	—
„ 3 · 10 ⁻³	—	—	62	—	—
Galactose 3 · 10 ⁻⁴	99	—	—	—	—
„ 10 ⁻³	133	—	—	—	—
„ 3 · 10 ⁻³	—	78	—	31	20
Fructose 10 ⁻²	—	29	—	77	37
	—	—	—	—	—

Table 5. *Sugar content in excised roots of different species.* The values give the content calculated as mg glucose/g fresh matter. For further explanations see Stenlid 1957 b, p. 925.

Plant species analysed	Time of pretreatment in distilled water, hours					
	0		24		48	
	Red. sugar	"Total" sugar	Red. sugar	"Total" sugar	Red. sugar	"Total" sugar
Wheat	8.1	9.4	5.1	5.7	2.5	3.1
Cucumber	5.2	9.9	1.4	2.8	0.9	1.6
Cress	4.0	4.8	—	—	—	—
Pea	20.2	25.4	—	—	—	—

Table 6, Exp. C that the growth in $5 \cdot 10^{-4}$ M galactose was 9.7 mm./24 hours and in $5 \cdot 10^{-4}$ M mannose 10.7 mm./24 hours. If the nutrient solution contained both $5 \cdot 10^{-4}$ M galactose and $5 \cdot 10^{-4}$ M mannose the growth was 16.7 mm./24 hours. The results must be interpreted as an example of mutual antagonism, *i.e.* mannose reduces the inhibitory effects of galactose and galactose reduces the inhibitory effects of mannose. In experiments with isolated tomato roots Ferguson *et al.* (1958 b) observed a similar mutual antagonism between the toxic sugars xylose, galactose, and mannose. The interpretation of their results is still more complicated as the solutions contained 1 per cent of sucrose in addition to the toxic sugars.

Many different possibilities of interaction exist when two toxic sugars (mannose and galactose) are given simultaneously to wheat roots. Some of

Table 6. *The effect of combinations of galactose and mannose upon the growth of wheat roots.* Growth expressed as mm/24 hours.

Exp.	Mannose added, M	Galactose added, M					
		0	$2 \cdot 10^{-4}$	$3 \cdot 10^{-4}$	$5 \cdot 10^{-4}$	$8 \cdot 10^{-4}$	10^{-3}
A	0	26.1	—	18.6	9.7	9.9	—
A	$3 \cdot 10^{-4}$	18.4	—	19.1	—	—	—
A	$5 \cdot 10^{-4}$	14.4	—	—	13.7	—	—
A	$8 \cdot 10^{-4}$	9.9	—	—	—	11.6	—
B	0	27.4	21.1	17.5	10.6	—	—
B	$3 \cdot 10^{-4}$	19.4	21.4	21.8	13.6	—	—
B	$5 \cdot 10^{-4}$	15.7	—	19.1	15.1	—	—
C	0	25.3	—	—	9.7	—	6.8
C	$5 \cdot 10^{-4}$	10.7	—	—	16.7	—	6.6
C	10^{-3}	8.1	—	—	8.9	—	6.0
D	0	26.8	—	—	14.7	11.6	—
D	$5 \cdot 10^{-4}$	12.0	—	—	16.2	13.6	—
D	$7 \cdot 10^{-4}$	9.7	—	—	14.2	13.6	—
E	0	29.5	23.4	—	16.3	—	—
E	$5 \cdot 10^{-4}$	12.9	16.7	—	21.4	—	—

Table 7. *The effect of combinations of galactose and 2-DG upon the growth of wheat roots.*
For further explanations see Table 6.

Exp.	2-DG added, M	Galactose added, M			
		0	$2 \cdot 10^{-4}$	$3 \cdot 10^{-4}$	$5 \cdot 10^{-4}$
A	0	29.5	23.4	—	16.3
A	$5 \cdot 10^{-5}$	8.7	15.6	—	18.7
B	0	28.3	20.6	18.8	10.8
B	$3 \cdot 10^{-5}$	13.3	22.5	20.3	12.3
B	$5 \cdot 10^{-5}$	8.3	20.2	13.2	8.5

them will be briefly discussed below. When a second sugar is added the concentration of the first is supposed to be unchanged. The total sugar content is thus supposed always to be higher in the mixture (cf. Tables 6 to 8).

1) *The growth inhibitions caused by galactose and mannose are identical.* An addition of mannose to a galactose solution should probably result in a growth intermediate between the growth in the simple sugar solutions if mannose goes in only at sites already occupied by galactose. If there are also free sites (which seems most probable if the growth inhibition is increased after addition of higher concentrations of galactose) an increased inhibition would be rather likely when mannose is added. It is near at hand to suppose that the simultaneous addition of two sugars with identical action works in the same direction as an increase in concentration of one of them. A complicating factor is that the degree of saturation giving the same per-centual inhibition may be very different for the two sugars.

2) *Galactose and mannose inhibit at two different sites and they have no toxic or antagonistic effects at the other site.* In this case the inhibition ought to be increased (or possibly unchanged) if a second sugar is added.

3) *The effects are caused at different sites and one of the sugars is a competing non-toxic substrate at the site of the other sugar.* In this case an increased or unchanged inhibition may occur if the sugars are added together, but an effect between those obtained in the one-sugar solutions is also conceivable. A complicating factor is that the competing sugar may either be an indifferent compound (which is not utilized in the reaction) only displacing the toxic sugar, or it may be directly used in the reaction.

4) *The toxic effects are caused at different sites and the sugars are mutual antagonists against each other.* The picture becomes very complicated and both increased inhibition, intermediate effects and an inhibition which is less pronounced than in any of the one-sugar solutions may appear. The real meaning of the mutual antagonism observed in roots is not easy to elucidate, but it seems most simple to postulate at least two different points where the sugars may effect the growth mechanism.

Table 8. *The effect of combinations of 2-DG and mannose upon the growth of wheat roots.*
For further explanations see Table 6.

Exp.	2-DG added, M	Mannose added, M		
		0	$3 \cdot 10^{-4}$	$5 \cdot 10^{-4}$
A	0	25.5	16.3	—
A	$2 \cdot 10^{-5}$	16.7	14.2	—
B	0	24.1	—	13.9
B	$3 \cdot 10^{-5}$	17.3	—	14.0
C	0	26.1	—	11.5
C	$4 \cdot 10^{-5}$	9.2	—	9.6

If it is assumed that mannose and galactose inhibit the growth process at two different sites it is not necessary to assume that the reversing effects are manifested at these growth sites themselves. The antagonistic effect of one or both of the sugars may be exerted on the absorption, transport or transformation of the toxic sugar. Such an interference may lead to decreased concentration of the toxic sugar at the active sites and thus to antagonistic effects.

Ferguson *et al.* (1958 b) report that there is no special antagonism of glucose against the galactose uptake in lucerne roots. The uptake of galactose is somewhat reduced from a mixed solution (0.2 per cent of each sugar) as compared to the absorption from a 0.2 per cent galactose solution, but not more than the uptake of glucose. Also in the present investigation some preliminary determinations of the sugar absorption from mixtures have been made. The total absorption of sugars from a solution with 1 mM mannose and 1 mM galactose exceeded the absorption from solutions containing 2 mM of one of the sugars. It seems thus as if there is no pronounced antagonism in the absorption mechanism, but without more accurate determinations it is impossible to decide to what extent complications during the uptake may contribute to the observed effects upon growth. In disks of radish roots glucose was found to inhibit galactose absorption (Säid and Fawzy 1949).

In experiments with combinations of 2-DG and galactose (Table 7) a similar mutual antagonism was found as between mannose and galactose. In mixtures of 2-DG and mannose, on the other hand, no clear signs of a mutual antagonism were observed (Table 8).

Comparing now the results obtained with *e.g.* cucumber and wheat roots, it is obvious that galactose is an antagonist towards mannose in both species, both as regards growth and chloride absorption (cf. next section). It is possible that the toxic effects of mannose and the reversal caused by galactose are of the same nature in both species in spite of their different reaction to galactose.

Galactose probably attacks the growth mechanism of wheat roots in another manner than mannose. The diverging effects of the two sugars upon the absorption of chloride and nitrate support such an interpretation. Mannose is antagonistic against galactose in the growth of wheat roots as the inhibitory effects of galactose are partly reversed by mannose.

From experiments with *Avena coleoptiles* Ordín and Bonner (1957) and Thimann *et al.* (1958) conclude that galactose is incorporated into polysaccharides. It interferes with the normal cellulose synthesis and seems to hinder the normal utilization of glucose. Cell elongation in the coleoptiles is inhibited without any signs of a disturbed respiration. This interpretation is consistent also with the results obtained with roots of wheat and other grasses.

As regards those roots, where growth is not inhibited by galactose, it must be left undecided if the galactose sensitive site is not present, or if galactose for some reason (*e.g.* conversion to glucose) is unable to inhibit this special point in the growth mechanism. The evidence for conversion of galactose to glucose in plants steadily increases (see Ginsburg *et al.* 1956, Neufeld *et al.* 1957) but it is still uncertain if the results are applicable upon the present data.

Chloride Absorption

Chloride absorption was determined as described in an earlier communication (Stenlid 1957 b). The roots used were 40–60 mm. long and had been grown for 3–4 days in Petri dishes in the dark at 22°–23°C.

In cucumber roots chloride absorption is inhibited by mannose, whereas glucose and galactose produce only weak effects. These two sugars and sucrose reverse the toxic effects obtained with mannose (see Tables 9 and 10). On the whole the results agree with those obtained with wheat roots. Some quantitative differences may be noted, however. No distinct stimulating effects of sugars upon chloride absorption have been observed in cucumber roots pretreated in aerated water. The mannose inhibition in cucumber roots is not very much increased at higher concentrations of mannose, and it seems as if about 50 per cent of the chloride accumulation is insensitive to mannose.

Cress roots show a vigorous accumulation of chloride ions; in freshly excised roots it is 2–3 times as rapid as in cucumber and wheat roots, when calculated on a dry matter basis. Quite as the roots of other species, cress roots give an enhanced chloride accumulation if the roots are pretreated in aerated water (Figure 3). The decline in accumulation capacity after continued aeration sets in earlier and is more pronounced than for wheat

Table 9. *The effect of glucose, galactose, mannose, and 2-DG upon chloride absorption in excised cucumber and pea roots, pretreated with distilled water. The values give the absorption in per cent of control without sugar.*

Addition of sugars, <i>M</i>	Time of pretreatment, hours						
	0	19—24		42—48		70—72	
	Cucumber	Cucumber	Pea	Cucumber	Pea	Cucumber	Pea
Mannose 3·10 ⁻⁴	74	—	—	—	—	—	—
„ 10 ⁻³	61	75	—	65	—	—	—
„ 3·10 ⁻³	64	49	55	52	—	50	—
„ 10 ⁻²	48	56	—	47	—	49	55
Galactose 3·10 ⁻³	87	—	87	—	85	—	90
„ 10 ⁻²	83	91	41	94	59	—	67
„ 10 ⁻² + Glucose 10 ⁻²	—	—	—	—	84	—	—
„ 10 ⁻² + „ 3·10 ⁻²	—	—	—	—	90	—	—
Glucose 3·10 ⁻³	88	—	114	—	—	—	—
„ 10 ⁻²	89	96	97	99	—	110	109
2-DG 10 ⁻³	—	—	44	—	—	—	44

and cucumber roots. A notable property of cress roots is that the accumulation of chloride in freshly excised roots is insensitive to mannose and 2-DG in concentrations up to $3 \cdot 10^{-2}$ *M* (Table 11). In roots which were pretreated during 24 hours a weak inhibition is obtained. As in cucumber roots the inhibition deviates from the one in wheat roots with regard to the response to increased concentration. In cress roots the inhibition is about the same in $3 \cdot 10^{-3}$ *M* as in $3 \cdot 10^{-2}$ *M* mannose. The mannose inhibition in pretreated cress roots is reversed by glucose and galactose (Table 10).

A possible interpretation of the results is that the endogenous sugar supply is sufficient to antagonize the externally supplied mannose in freshly excised roots but not in pretreated roots. The results with nitrate absorption (Table 15) agree with such an interpretation.

Table 10. *Reversing effect of galactose, glucose, and sucrose upon the mannose inhibition of chloride absorption in cucumber and cress roots. Cucumber roots freshly excised, cress roots pretreated in distilled water 24 hours. The absorption is expressed as per cent of control without sugar.*

Plant species	Mannose conc., <i>M</i>	Reversing sugar added, <i>M</i>							
		0	Galactose		Glucose			Sucrose	
			$3 \cdot 10^{-3}$	10^{-2}	10^{-3}	$3 \cdot 10^{-3}$	10^{-2}	$3 \cdot 10^{-3}$	10^{-2}
Cress	10^{-3}	78	94	—	—	90	—	—	—
"	$3 \cdot 10^{-3}$	66	—	—	—	107	106	—	—
"	10^{-2}	62	—	—	—	99	—	—	—
Cucumber	10^{-3}	63	65	99	—	—	—	84	82
"	$3 \cdot 10^{-3}$	52	—	—	63	81	82	—	—

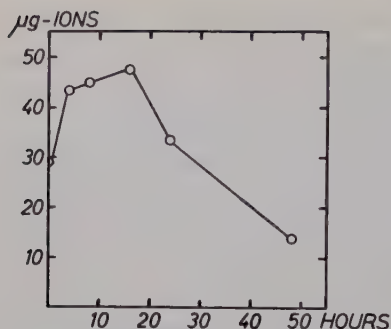


Figure 3. The capacity of chloride absorption in excised cress roots pretreated with distilled water. The absorption is given as $\mu\text{g-ions}/4$ hours and 100 mg dry matter.

In experiments with cucumber roots no difference in the sensitivity to mannose inhibition was observed between the differently pretreated roots (Table 9) if the inhibition is expressed as per cent of the absorption in the controls. As the absorption of the control is changed, a comparison between different types of roots is difficult, however.

In wheat roots, the inhibitory effect of mannose is less pronounced in pretreated roots (Table 12), whereas the inhibition caused by 2-DG is more distinct in roots pretreated for 72 to 96 hours than in freshly excised roots. In pretreated roots 2-DG is therefore the more toxic of the two sugars in contrast to what is found in freshly excised roots (cf. Stenlid 1954). It is difficult to draw any conclusions from this conversion in the sensitivity to the two sugars as many complicating factors (*e.g.* altered absorption rate of the sugars) may be responsible for the results. In any case the simple interpretation which was suggested for cress roots is not possible to apply for wheat roots. It is not possible to find any relation between the sugar

Table 11. The effect of some sugars upon chloride uptake in excised cress roots pretreated with distilled water. The values give the absorption in per cent of control without sugar.

Sugar added, M	Time of pretreatment, hours		
	0	20—24	44—48
Mannose 10^{-3}	94	72	—
" $3 \cdot 10^{-3}$	96	63	—
" 10^{-2}	94	60	40
" $3 \cdot 10^{-2}$	108	66	—
Glucose 10^{-2}	—	103	127
" $3 \cdot 10^{-2}$	—	98	—
2-DG 10^{-3}	110	76	—
" $3 \cdot 10^{-3}$	113	58	—
" 10^{-2}	119	—	—
Galactose 10^{-2}	105	97	112
" $3 \cdot 10^{-2}$	115	97	—
D-xylose $3 \cdot 10^{-2}$	—	101	—

Table 12. *The effect of different sugars upon chloride absorption in wheat roots pretreated with distilled water for different time periods.* The values for the control given as $\mu\text{g-ions/4 hours and 100 mg dry matter}$, the other values as per cent of the control. Every value is the mean of three or six different tubes.

Addition to the control, M	Time of pretreatment, hours				
	0	24	48	72	96
0	11.4	29.4	31.9	21.1	12.8
Glucose $3 \cdot 10^{-3}$	83	98	119	137	152
Galactose $3 \cdot 10^{-3}$	113	124	114	117	142
Mannose 10^{-3}	30	61	66	59	45
" $3 \cdot 10^{-3}$	13	30	52	51	—
2-DG 10^{-3}	51	76	61	39	19

content and the sensitivity to mannose, as cress roots have a lower content of sugars than wheat and cucumber roots (Table 5). Both the availability of the endogenous sugars and the absorption of the inhibitory sugars may be different in different roots, however. Nor is it sure that the chloride absorbing mechanism is the same in various roots.

In the pea roots chloride absorption is rather slow and pretreatment of the roots is necessary to obtain any chloride accumulation at all. Pea roots differ from all other roots tested in that the chloride absorption is distinctly inhibited by galactose (Table 9). It should be noted that galactose is more toxic to chloride accumulation than to growth. The growth experiments were performed with intact plants, however, which certainly have a more abundant supply of endogenous sugars. Mannose and 2-DG are likewise toxic to chloride accumulation in pea roots. Quite as in the growth experiments relatively high concentrations of the sugars are necessary to cause the inhibitory effects. The galactose inhibition is reversed by glucose (Table 9).

Nitrate Absorption

The effect of glucose and galactose upon nitrate uptake in wheat roots was reported in an earlier paper (Stenlid 1957 b). In Table 13 some results with other sugars are given. For the methods used see Stenlid 1957 b. Mannose and 2-DG are both distinctly toxic to nitrate absorption. Glucose and galactose reverse the mannose inhibition.

The assimilation of nitrate may be affected in another way than the absorption (cf. Burström 1948, 1949) and a disturbed protein synthesis (*e.g.* in the presence of galactose) is conceivable in spite of a quantitatively normal absorption. Some experiments were made in which the absorption and assimilation of nitrate was calculated from determinations in both the solution

Table 13. *The effect of some sugars upon nitrate accumulation in excised wheat roots. In some experiments the reversing effect of glucose and galactose upon the mannose inhibition was studied. The values give the accumulation in per cent of control without sugar. Every value is the mean of at least six tubes from at least two different experiments.*

Sugar added, <i>M</i>	Reversing sugar added, <i>M</i>								
	Freshly excised roots				Roots pretreated in distilled water 20–24 hours				
	0	Galac- tose 10 ⁻³	Glucose		0	Galactose		Glucose	
			3 · 10 ⁻⁴	10 ⁻³		10 ⁻³	3 · 10 ⁻³	10 ⁻⁴	3 · 10 ⁻⁴ 10 ⁻³
Mannose 3 · 10 ⁻⁴	79	—	—	—	88	—	—	—	—
" 10 ⁻³	30	58	75	104	30	52	80	53	100 120
" 3 · 10 ⁻³	19	—	—	—	—	—	—	—	—
2-DG 3 · 10 ⁻⁴	75	—	—	—	40	—	—	—	—
" 10 ⁻³	33	—	—	—	17	—	—	—	—
Sucrose 10 ⁻³	112	—	—	—	173	—	—	—	—
D-xylose 10 ⁻³	—	—	—	—	104	—	—	—	—

and the roots (Table 14). The effects upon assimilation and accumulation are rather similar and these experiments give no clue to any abnormal nitrate assimilation in wheat roots in the presence of galactose. It is, of course, nevertheless possible that some anomalous products are formed when glucose is replaced by galactose and that these products give rise to the inhibition of growth caused by galactose. Sucrose, which is toxic to wheat roots under certain conditions (Burström 1941) gives a pronounced increase of both assimilation and uptake of nitrate (Tables 13 and 14).

Cucumber roots (Table 15) differ only little from wheat roots. The inhibition by galactose in freshly excised roots seems to be absent in cucumber roots, however. No decrease in absorption capacity was observed in roots pretreated with distilled water for 24 hours (cf. the effect of pretreatment

Table 14. *The effect of some sugars upon absorption and assimilation of nitrate in excised wheat roots. Every value is the mean of determinations from 3 tubes.*

Sugar added, <i>M</i>	μg-ions nitrate/4 hours and 100 mg. dry matter					
	Freshly excised roots		Roots pretreated 20–24 hours		Roots pretreated 44–48 hours	
	Absorp.	Assim.	Absorp.	Assim.	Absorp.	Assim.
0	10.4	3.8	7.9	2.4	4.7	0.4
Glucose 10 ⁻³	—	—	10.1	5.1	—	—
" 2 · 10 ⁻³	—	—	—	—	9.1	2.9
Galactose 10 ⁻³	—	—	8.2	3.4	8.1	2.9
" 2 · 10 ⁻³	7.0	2.7	—	—	—	—
Mannose 2 · 10 ⁻³	2.6	0.9	—	—	—	—
Fructose 10 ⁻³	—	—	8.5	2.9	—	—
Sucrose 10 ⁻³	—	—	15.0	8.5	11.0	5.2

Table 15. *The effect of some sugars upon nitrate absorption in cucumber, pea and cress roots. For further explanations see Table 13.*

Sugar added, <i>M</i>	Cress roots		Cucumber roots		Pea roots	
	Freshly excised	Pretreated 20—24 h.	Freshly excised	Pretreated 20—24 h.	Pretreated	
					20—24 h.	42—48 h.
Glucose 10 ⁻³	115	178	93	123	128	208
" 3 · 10 ⁻³	137	173	105	121	136	—
Galactose 10 ⁻³	—	—	129	136	—	—
" 3 · 10 ⁻³	120	160	92	118	139	144
Mannose 10 ⁻³	84	59	76	48	—	—
" 3 · 10 ⁻³	69	—	—	—	—	—
2-DG 10 ⁻³	93	51	—	—	—	—

upon wheat roots, Stenlid 1957 b, and upon phosphate absorption, Stenlid 1959).

Cress roots too, behave similarly as wheat roots (Table 15). Comparing the results with those obtained in the chloride experiments it is seen that more distinct results are obtained with nitrate, which is consistent with the view that the carbohydrate requirement of nitrate absorption is larger.

With pea roots only glucose and galactose have been tested and they both stimulate nitrate uptake in pretreated roots. As galactose inhibited chloride absorption (cf. Table 9) it seems as if the relation of ion absorption to galactose in pea roots diverges from that in other roots. It is possible that this is connected with the special physiology of leguminous plants.

Oxygen Uptake

The oxygen uptake was determined in a Warburg-Barcroft apparatus in 0.01 *M* phosphate buffer with 0.001 *M* CaCl₂. The initial rates of oxygen uptake were determined during two hours before the sugars were added. The oxygen uptake after the addition of sugars is expressed as per cent of the control (a correction is applied for differences in the initial rates). 4—10 roots (total length about 40 mm.; the number of roots was different for different species) cut into pieces of 12—15 mm. were used in each vessel. Each value is calculated from determinations in 3 control vessels and 3 vessels with sugar.

In the reaction of oxygen uptake to the addition of different sugars little variance has been observed between different species. Mannose inhibited oxygen uptake in all species tested, and in all cases only a partial inhibition was obtained, which was much less pronounced than the effects upon ion uptake and growth. The results with mannose in freshly excised roots for barley, oats, rye, maize, flax, cress, and pea agree with those for wheat and cucumber roots in Table 16. Glucose, fructose, galactose, and sucrose as a

Table 16. *The effect of some sugars upon the oxygen consumption in excised roots of wheat and cucumber. The values 0—60, 60—120 and 120—180 denote the period (in minutes) after the addition of sugar during which oxygen uptake was determined. The values are expressed as per cent of the control. For further explanations see p. 231.*

Roots used	Addition to the control 0.05 M	Time of pretreatment in distilled water, hours											
		0			24			48			72		
		0—60	60—120	120—180	0—60	60—120	120—180	0—60	60—120	120—180	0—60	60—120	120—180
Wheat	Glucose	115	117	128	130	137	155	143	170	191	170	240	258
"	Galactose	103	101	103	110	113	118	130	136	143	165	224	220
"	Mannose	91	69	67	88	64	74	118	106	108	123	151	158
"	2-DG	84	68	70	—	—	—	—	—	—	138	151	135
Cucumber	Glucose	115	111	111	—	—	—	178	167	240	137	153	191
"	Galactose	120	117	123	—	—	—	149	153	212	146	145	165
"	Mannose	97	81	80	—	—	—	101	95	112	127	118	135

rule gave weak positive effects. These positive effects are much more distinct in roots which have been pretreated in aerated distilled water (in Table 16 experiments with wheat and cucumber roots are recorded). A salient result is that in roots, which have been pretreated for 48 hours or more, mannose and 2-DG give positive effects, although not equally conspicuous as the non-toxic sugars.

The positive effects of mannose in pretreated roots together with the incomplete inhibition in freshly excised roots can be interpreted in different ways, and some possible explanations are suggested below.

1) Mannose is used in the root respiration, but with a lower rate than the normal substrate. When the supply of normal substrate is high, mannose competes with it and inhibits the respiration. If the supply is low and sugar (carbohydrate) concentration is a limiting factor, competition is less prominent and positive effects are obtained (cf. the effects of galactose upon nitrate uptake and of mannose upon phosphate uptake in wheat roots; Stenlid 1957 b, 1959). The different relative sensitivity of chloride absorption to mannose and 2-DG in freshly excised and in pretreated wheat roots can scarcely be explained only by a decreased sugar content. It seems obvious that other changes are also important for the reaction to the added sugars.

2) The normal respiration is composed of different pathways, the relative importance of which is different in starved and non-starved roots. If mannose does not inhibit all of the pathways but is utilized in some of them the results may be explained, provided that the mannose-inhibited mechanism is less dominant in pretreated roots.

It is not possible to discuss the complicated question of the respiratory changes in starved roots in detail (see James 1953, Girtton 1958). Other sub-

strates than carbohydrates may be utilized, and an interference of the pentose phosphate pathway, which seems to be common in plants (see Axelrod and Beevers 1956, Albaum 1958) is also possible. It has been shown that the pentose phosphate pathway is more important in old tissues whereas the glycolytic route is predominant in juvenile tissues (Gibbs and Beevers 1955). A similar change is possible between freshly excised and pretreated roots.

3) The positive effects of mannose in pretreated roots are indirect, and the respiratory substrate is not mannose, but some endogenous substance, the degradation of which is increased through the addition of mannose. If mannose is converted to mannose phosphate, ADP may be formed giving rise to an increased respiration.

It is difficult to decide between the different possibilities without further data (*e.g.* experiments with labelled respiratory substrates). The stimulation of oxygen uptake may quite well be of different nature for different sugars. In this connection it can be pointed out that glucose and galactose increase the absorption of both chloride, nitrate and phosphate in pretreated roots. Mannose and 2-DG stimulate only the phosphate uptake (see Stenlid 1959) and the effects of sugars upon phosphate absorption and oxygen uptake agree fairly well. As the chloride absorption is inhibited by mannose and 2-DG even in pretreated roots it seems as if the respiratory stimulation caused by these sugars is not utilizable for the absorption of chloride ions.

Summary

The effect of different sugars upon growth, chloride absorption, nitrate absorption, and oxygen uptake has been studied in roots of different plant species. Excised roots from 3—4 days old seedlings were used, except for the growth experiments, which were performed with intact plant roots.

Root growth of all species tested was inhibited by mannose and 2-desoxy-D-glucose (2-DG). Galactose inhibited growth in some species (*e.g.* wheat, rye, barley, oats), but was non-inhibitory to other species (*e.g.* cucumber and cress).

The growth inhibitions were reversed by glucose and in cress and cucumber roots also by galactose.

In wheat roots the inhibitory sugars galactose and mannose behave as mutual antagonists, and it is concluded that they have different modes of inhibitory action.

As a rule chloride and nitrate absorption are inhibited by mannose and 2-DG and stimulated by glucose and galactose. In pea roots galactose is inhibitory to chloride absorption.

In freshly excised roots mannose and 2-DG inhibit oxygen uptake, but in roots of wheat and cucumber pretreated in distilled water during 48 hours or more positive effects of the two sugars upon respiration are obtained.

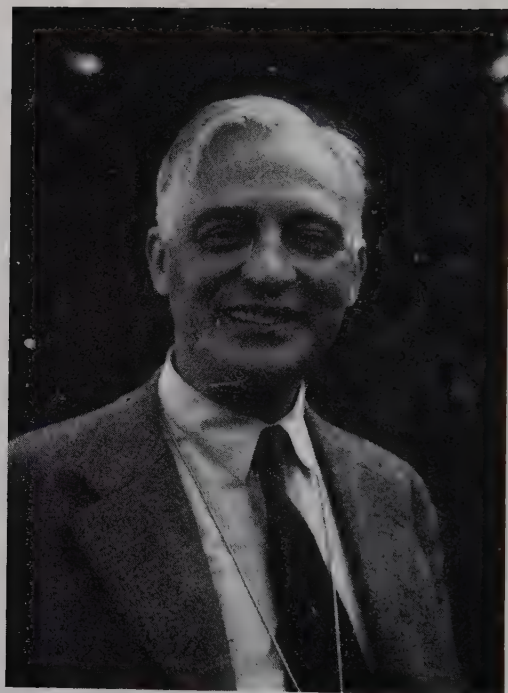
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PROFESSOR DETLEV MÜLLER

Quantitative Changes of Growth-Promoting and Inhibiting Substances in the Potato Tubers Treated with Rindite

(Physiological Study of the Potato XV)

By

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(Received July 31, 1958)

Introduction

The contents of growth-promoting and inhibiting substances in the potato tubers has been thoroughly studied by Hemberg (1942, 1943, 1946, 1947, 1949, 1952, 1954). Attempts to learn the growth substances found in the potatoes have also been made earlier, *e.g.* Jahnel (1937, 1939) and Lucas (1939) extracted the growth substances directly from the potato tissues with agar blocks. Guthrie (1939), Boysen-Jensen (1941), Dostal (1942), and Michener (1942) extracted the potatoes with ether, however, only Boysen-Jensen purified the extract before employing it for biological test.

Hemberg demonstrated three substances, determining the resting and sprouting of the tubers, namely, an acidic and a neutral growth substance, further an acidic inhibiting substance soluble in water and ether which can be separated from the growth substance by diffusion. These substances were extracted from the skin, cortex, vascular cylinder, and from the pith with ether, and their appearance in different tissues of the potato at the various periods examined.

Blommaert (1954) demonstrated with a paper chromatographical method connected with a biological test that the acidic inhibiting substance of resting potato tubers disappears before sprouting and then the increase of the content of free auxins and of their precursors (indolylacetaldehyde IAAd,

indolylpyruvic acid IPA, indolylacetonitrile IAN etc.) commences. These experiments, however, referred merely to the spontaneously sprouting tubers.

In our country Varga and Ferenczy (1957) dealt with the growth-promoting and inhibiting substances of the potato, variety "Korai sárga" ("early yellow"). They studied the changes of the ether-extractable acidic growth-promoting and inhibiting substances due to rindite-treatment with paper chromatography and subsequently with Avena test. The rindite is a mixture of ethylene chlorhydrine, ethylene dichloride and carbon tetrachloride (7 : 3 : 1), which very rapidly repeals the dormancy of the new potatoes (Denny, 1945). Varga and Ferenczy established that the decomposition of the inhibiting substances and the increase of the content of growth substances occurred considerably earlier in the rindite-treated tubers than in the spontaneously sprouting controls.

In order to study the metabolism of the growth substances of potatoes in detail, it seemed necessary to examine the quantitative changes in growth-promoting and inhibiting substances in the sectors, differing physiologically and also structurally, of the tubers in the different phases of the sprouting, further, to compare the results in case of the rindite-treated "young" and the spontaneously sprouting "old" tubers.

Methods

New tubers treated with 0.8 ml./kg. rindite for 48 hours after a 24-hour ventilation have been planted together with the controls in wet sand. The growth-promoting and inhibiting substances were extracted on the 1, 8, 15, 22, and 29 days following treatment with the method later described. In each case 20 tubers were employed for the analysis, the sprouts, however, previously broken off.

The potato tubers were cut in four sectors (apical "A", basal "C", girdle "B", and pith "D") then the different parts, except the pith, were finely ground and extracted with peroxidefree ether according to the combined procedure of Bonde and Larsen.

The ether fractions were dropped on Sch. et Sch. 2043 b paper and run in isopropanol-ammonia-water (10 : 1 : 1) solvent with ascending method (Bennet-Clark and Kefford 1953, Bennet-Clark, Tambiah, Kefford 1952).

Reaching the front line, at 20 cm. distance, the running was stopped, the paper dried and after observation under analytical UV-lamp, it was examined with biological test.

The native auxin content of the coleoptile sections was decreased by soaking them in pure water before the test. The chromatograms were cut transversally into 20 strips, 1 cm. wide. Each strip was placed in a glass tube and eluted with 1 ml. bidistilled water. Ten subapical coleoptile sections, 5 mm. long, were then placed into each sample tube (Bentley and Housley 1954, Kefford 1955 a, b, Larsen 1955, Nitsch 1955) and floated on the eluate. Incubation lasted for 24 hours at 23°C. in a dark chamber saturated with humidity (Larsen).

Hereafter the images of the coleoptile sections were projected at tenfold magnification with a photographic enlarger and their length was measured with a transparent, flexible ruler. The average values of growth reactions were calculated as percentage related to the elongation of the controls.

For biological test *Avena sativa* L., "Fleischmann", was used, made available by the Plant Breeding Experimental Institute at Tápíószele.

Experimental Results

Hemberg's examinations (1946, 1947, 1949, 1952) show that the inhibiting substances due to the rindite treatment quickly disappear during the sprouting while the quantity of the growth-substances increases. Varga and Ferenczy (1957) have presumed that some inhibiting substance is present during the sprouting, the effect of which, however, is insignificant. To clear up the contradictions, I concerned myself merely with the quantitative changes of the β -indolylacetic acid (IAA) and of the so-called β -inhibitor. Changes of the IAA (Rf 0.35 to 0.40) and of the β -inhibitor (Rf 0.55 to 0.5) content, measured during the sprouting, are shown in Figure 1.

On the first day following treatment, when the tubers practically are still "resting", the elongation of the coleoptiles amounts to 5 % in the eluate of the IAA spot of sector "A". On the 8th day the results are the same; on the 15th day, however, the maximal stimulation was noted (19 % growth surplus). Then the growth stimulation was gradually reduced, on the 22d day it was 15 % and on the 29th day 14 %.

In the eluate of the IAA spot of sector "B" no growth stimulation was noted on the first day. Beginning from the 8th day the auxin content is gradually increasing, producing 4, 5, 9 and 18 % growth surplus.

The conditions in sector "C" is similar to "B", herein the quantity of IAA also gradually increases.

The quantity of inhibiting substances (see Figure 1, No. 2) tend to decrease. During the sprouting, the rate of the initial inhibition decreases in all the sectors, however, in "B" and "C" this is less characteristic. Somewhat marked is the result on the 29th day which consistently shows a strong inhibition.

The quantity of growth substances in the control tubers was examined only on the first and the last day of the experiment as the buds on the untreated tubers began to sprout about the end of the examinations. On the first day merely a fairly mild growth stimulation was noted in sector "A" while on the 29th day all the sectors contained IAA in measurable quantity. The inhibition was considerable in all the sectors at the beginning as well as at the end of the experiment.

Bennet-Clark and Kefford 1953, and Blommaert 1954). These substances, being on the one hand of lesser significance as compared to the active area at Rf 0.35 to 0.40, and on the other, could not be included in my present objective, were also left out of consideration.

Remarkable is the broad inhibiting zone appearing on the chromatograms at Rf 0.55 to 0.75. This inhibiting zone was observed on the chromatograms of resting potatoes first by Blommaert (1954). At similar Rf value as this, growth-inhibiting substances were demonstrated also from different parts of plants. Varga and Ferenczy also found, at Rf 0.65 in the tubers of variety "early-yellow" treated with rindite, this substance which is considered to be identical with that described in the literature as " β -inhibitor" (Bennet-Clark and Kefford 1953).

Figure 1 proves that the quantity of IAA present in the new tubers at the beginning of the experiment, rapidly increases up to the 15th day in sector "A", followed by a decrease, while in sectors "B" and "C" it increases all the time. The decrease of the IAA content in sector "A", related to the development of the buds, supports rightly the assumption that this fall is to be attributed to the utilization.

It is noteworthy that the β -inhibitor, demonstrable in considerable quantity at the beginning of the experiment and later rapidly decreasing in all the sectors, begins to rise again from the 29th day. These observations seemingly contradict to the results of Varga and Ferenczy who noted a decrease of the IAA content in tubers treated with rindite, whereas the β -inhibitor completely disappeared. These discrepancies decrease considerably in view of the fact that the above mentioned authors examined only the cortex and no longer than 9 days, in contrast to my observations lasting 29 days; moreover, in the present work also other tissues of the tuber were examined. This circumstance explains the contradiction regarding the β -inhibitor (Figure 1, sectors "B" and "C"); however, it does not explain the considerable decrease of the IAA content. It is notable, that the variety of "early yellow", examined by Varga and Ferenczy, reacts more quickly to the rindite-treatment than that of "Kisvárda rose", examined by the author, and so it may be assumed that the different experimental results are due to the different behaviour of the two varieties.

The untreated (control) new tubers contained a considerable amount of β -inhibitor (Figure 1, No. 2) which may completely inhibit the action of IAA present in relatively small quantity, consequently, the sprouting could not begin.

As no own analytical data concerning the spontaneously sprouting tubers were available for comparison, I used the above-mentioned works of Hemberg and Blommaert as well as of Varga and Ferenczy (in our Institute).

According to these authors the acidic inhibitors in the spontaneously sprouting tubers disappear, the rise of the IAA content is, however, constant. As it has been shown, these processes are different in the new tubers treated with rindite. In these tubers the IAA content is rapidly rising at the beginning, then gradually decreases from the 15 day (the time of the vigorous commencement of the sprouting), however, it remains at a higher level than the initial one (Figure 1, No. 1). The rise of the IAA content in the control tubers is significantly less during the experiment (Figure 1, No. 2).

According to the above-mentioned authors the β -inhibitor gradually and completely disappears in the spontaneously sprouting tubers, however, in those treated with rindite its concentration only significantly decreases and increases again on the 29th day (Figure 1, No. 1). The change in the untreated tubers are insignificant (Figure 1, No. 2).

Hemberg observed (1949, 1950) the presence of free auxin in tubers not quite ripe at the beginning of the rest period. This statement tallies with the graph of Figure 1, No. 2. According to Hemberg, this free auxin is transformed into precursors in the autumn. In spring, before sprouting, the auxin content increases in the middle of the tubers, and auxin migrates into the cortex here causing a rise of the auxin concentration.

Summary

1. The quantity of the growth-promoting substances in new tubers, following rindite-treatment, rapidly increases, while that of inhibiting substances decreases. The rise of the IAA content in the apical part of the tubers is followed by a decrease on the 15th day due to the development of the buds, whereas in the sectors "B" and "C", where the sprouting is slower or even very often fails, the IAA content is constantly rising.

2. The β -inhibitor, present in considerable quantity at the beginning of the experiment, rapidly decreases in all the sectors of the rindite-treated tubers but rises again on the 29th day.

3. The rise of the IAA content in the spontaneously sprouting tubers is slower but constant while the inhibiting substances entirely disappear (Hemberg, Blommaert, Varga, Ferenczy).

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The Nature of the Germination Inhibitors Present in Dispersal Units of *Zygophyllum dumosum* and *Trigonella arabica*

By

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The presence of germination inhibitors in many fruits, seeds and plant extracts has long been known and has recently been reviewed by Evenari (1949) and by Koller (1954, 1955). It is the aim of the present work to try to identify chemically such inhibitors contained in the dispersal units of *Zygophyllum dumosum* and *Trigonella arabica*, where they have been stated to be present.

Methods

Water extracts of the dispersal units were made by placing them in water at 30°C overnight. The water is then decanted and filtered through a Büchner funnel lined with a few layers of gauze, cotton wool and a layer of celite. The water extract and also all subsequent extracts were tested for their inhibitory action towards lettuce seeds. For all the germination tests lettuce seed variety Progress was used. The seeds were germinated in water or the extracts at various concentrations in petri dishes in the dark at 26°C. In large petri dishes (9 cm. diameter) 3 ml. of solution and about 100 seeds were used, in small ones (4 cm. diameter) 1 ml. and about 50 seeds. The data are given in one of the two ways: either the concentration (grams of dry dispersal units/ml.) of test solution, causing 100 % inhibition is given, or the % germination at a certain concentration is given as a % of the germination in water. The latter was usually 80—90 %.

As germination inhibition is often due to osmotic pressure of the solution the osmotic pressure of all extracts was determined cryoscopically. The effect of osmotic pressure on the germination of lettuce seeds was determined by using solutions of

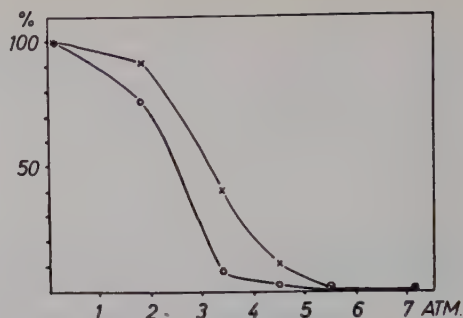
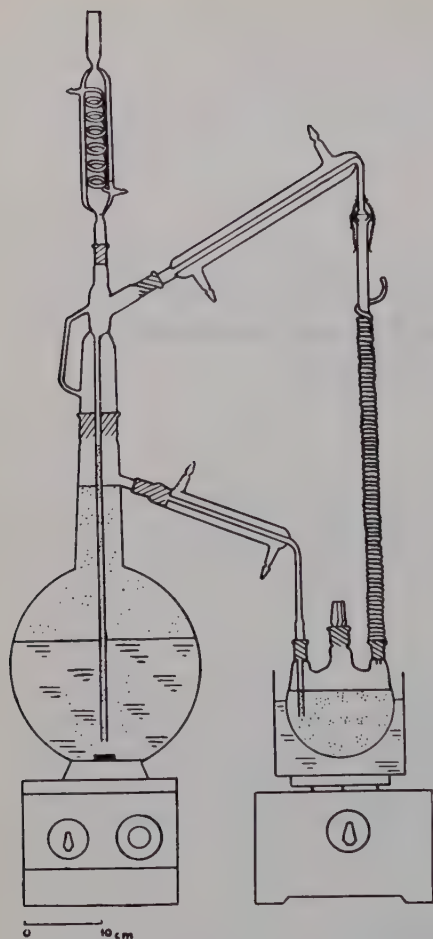


Figure 1. The relation between osmotic pressure and germination inhibition of crude *Zygo-phyl-lum* extracts and solutions of sodium chloride.

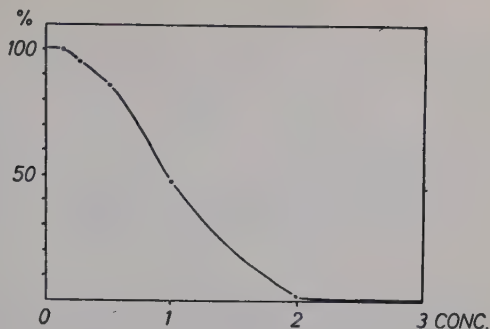
○—○ *Zygo-phyl-lum* extract
×—× NaCl solution

Figure 2. Liquid-liquid Soxhlet extraction apparatus for extracting large volumes of water with ether.

NaCl of various tonicity. The results given in Figure 1 were used as a reference curve for determining the effect of osmotic pressure of various extracts on germination. The results indicate that an osmotic pressure of six atmospheres is sufficient to inhibit completely the germination of the lettuce seeds used. The pH of the solutions to be tested was always checked so as to be between pH 4 and 8 so as to avoid effects of pH on germination. All chemicals, and especially solvents were tested for the presence of inhibitors. In many cases it was found that on concentrating large volumes of solvents inhibitors were present in the residues. Such solvents were not used.

Generally, during the purification of the inhibitors the water extracts were extracted with ether. Normal procedure would require extraction of the water three times with 2/10 of its volume of ether. As this was extremely inconvenient, a large liquid-liquid Soxhlet extraction apparatus was designed, in which five to six litres of aqueous extract could be treated (Figure 2). The solution was stirred during extraction with a magnetic stirrer. During extraction care must be taken to avoid the formation of gels or emulsions.

Figure 3. *The relation between concentration of partially purified inhibitor extracted from Zygothallum and its germination inhibition. The concentration of inhibitor is given as extracts of g. of Zygothallum dispersal units/ml. of test solution.*



Results and Discussion

As Koller (1955) has reported on the inhibitory properties of *Zygothallum* extracts, these were investigated in more detail. Figure 1 shows the relation between the osmotic pressure of aqueous *Zygothallum* dispersal units extracts and inhibition of germination of lettuce seeds. A comparison of this with the data for NaCl of equal osmotic pressure in Figure 1 shows that the crude *Zygothallum* extracts inhibit slightly more at the various osmotic pressures than would be expected. This indicates that some other factor contributes to the inhibition. Aqueous extracts were therefore extracted with ether. This ether extract was tested for inhibitory activity; this was done by evaporating the solvent and dissolving the residue in water and testing this solution for inhibitory action. This procedure was adopted at all subsequent stages. Inhibitory activity was found in the ether solution. For further purification the ethereal solution was concentrated on filter paper. The filter paper was eluted successively with petroleum ether, ether and finally water. These extracts were tested for germination inhibition; the inhibitory activity was found in the ethereal extract and not in the petroleum ether or the water extracts. It will be seen that the ethereal extract contains about 1/10 of the activity present in the original water extract (Table 1). The inhibitory substance present apparently contributes to some extent to the inhibition in the original extract. In the presence of the solutes causing osmotic inhibition, the trace of inhibitor can evidently cause the observed shift in the curve of inhibition as a function of osmotic pressure.

Small amounts of an inhibitor can apparently markedly modify osmotic inhibition. This is shown by the effect of the partially purified inhibitor on the inhibition caused by solutions of sodium chloride. Extracts of *Zygothallum* were prepared, extracted with ether and the ether passed through a magnesium carbonate-light column. The ether was evaporated to dryness and the residue taken up in hot distilled water and brought to pH 7.0. The

Table 1. *Effect of extracts of Zygophyllum on the germination of lettuce.*

Solution tested	Concentration of dispersal units causing complete inhibition (gm./ml. final solution)
Initial water extract	0.57
Crude ethereal extract	5.0
Ether eluate of extract on filter paper	5.0

effect of the inhibitor alone on germination is shown in Figure 3. Figure 4 shows the effect on germination of two levels of the inhibitor in the presence of various concentrations of NaCl. It will be seen that combinations of inhibitor and NaCl can in certain concentrations depress germination considerably more than either alone.

As a further object of study, *Trigonella arabica* was selected. Koller (1954) has shown that seeds of *Trigonella arabica* do not germinate within the dispersal unit. The dispersal units consist of the seeds inside their fruits. This suggested the existence of an inhibitor within the dispersal unit. Preliminary experiments showed that an inhibitor was indeed present in the dispersal unit. In addition, osmotic effects contribute about 50 % to the inhibitory action of water extracts of *Trigonella* dispersal units.

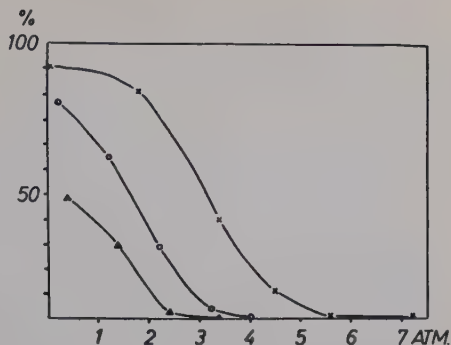
Ether extraction of the aqueous extract of *Trigonella* dispersal units was tried at various pHs. The ether extracts were then evaporated and the residue taken up in water and tested for activity (Table 2). As will be seen from Table 2, it is possible to extract an inhibitor from the acidified aqueous solutions which has about 20 % of the total inhibitory activity of the crude extract.

This inhibitor could be chromatographed on light magnesium carbonate or on alumina from which it can be eluted with ether.

The inhibitor was purified by the following procedure: 100 g. lots of dispersal units of *Trigonella* were extracted with one litre of water/100 g. at 70–90°C for ten minutes. The extract was filtered and the residue re-extracted with hot water for twenty minutes. The combined extracts were filtered, acidified to pH 5.5 and extracted with ether by the large Soxhlet procedure (Figure 2). The ethereal solution was passed through an alumina column. The solution coming from the column contained the inhibitor. The ether extract was dried on filter paper. The paper was extracted with petroleum ether in a standard Soxhlet apparatus, giving a partially purified extract. This was passed through a light magnesium carbonate column. The inhibitor was eluted from the column with petroleum ether. The petroleum ether extract was chromatographed on filter paper using water as the solvent. The chromatogram was cut and the location of the inhibitor determined by germination tests. The main activity was located around Rf 0.7. At this Rf value

Figure 4. Germination % as a function of osmotic pressure of NaCl solutions containing various concentrations of the partially purified inhibitor extracted from *Zygophyllum*.

- ×-× NaCl solution
 ○-○ NaCl+0.5 g./ml. inhibitor
 △-△ NaCl+1.0 g./ml. inhibitor



only one spot was present on the chromatogram. This spot gave the following reactions: It has photoactivated yellow-green fluorescence when irradiated with U.V. after spraying with 0.4 N NaOH. No fluorescence was observed without pretreatment with NaOH nor was ammonia vapour effective in causing fluorescence. The active spot gave a diazo reaction with sulphanilic acid only after treatment with 0.4 N NaOH. With *p*-nitro aniline, the substance gave a yellow spot when pretreated with 0.4 N NaOH. This spot changed to brown-violet after exposure to ammonia vapour. The active spot also reacted with aqueous 0.5 % potassium permanganate giving a yellow colour.

All these reactions indicate that the active substance is coumarin, being in accord with the behaviour described for coumarin, (Goodwin and Kavanagh 1950, Swain 1953). To establish this finally, the unknown compound was compared to coumarin by chromatography on paper. The unknown compound alone, coumarin alone and a mixture of two were run on paper in three different solvents. In all cases only one spot appeared from the mixture at the same R_f as coumarin and the unknown. In water the spots occurred between R_f 0.63—0.67, in ethyl acetate-HCl and in ethyl acetate-ammonia the spots occurred near the solvent front. Swain (1953) records R_f s of 0.67 for coumarin in water and at 0.95 in ethyl acetate-HCl and ethyl acetate-ammonia.

Table 2. Effect of pH of ether extraction on recovery of inhibitor for *Trigonella arabica*.

Solution	Concentration of solution Trigonella dispersal units mg./ml.	pH of extraction	Per cent germination
Crude aqueous extract O.P.~2.5 ..	0.1	no extraction	0
Acid ethereal extract	0.5	2	0
Neutral ethereal extract	0.5	6	1
Basic ethereal extract	0.5	10	34

The identity of the spots on the chromatograms was also confirmed by the tests previously described.

It can be concluded that the active compounds present in hot water extracts of *Trigonella arabica* dispersal units is coumarin. These dispersal units in addition cause germination inhibition by osmotic effects. During the isolation procedure other compounds causing milder inhibition were also observed. As their activity was very weak they were not further investigated. It must be pointed out that full recovery of the inhibitor was not obtained, the crude extracts inhibiting at lower concentrations than the purified ones.

In the light of the identification of the inhibitor in *Trigonella* the inhibitor in *Zygophyllum* was tested. It could be conclusively shown that it was not coumarin.

Summary

The nature of the germination inhibitors present in *Zygophyllum dumosum* and *Trigonella arabica* dispersal units was studied. In *Zygophyllum* practically all the inhibition was caused by osmotic effects which were modified by an ether soluble inhibitor. In *Trigonella* about half the germination inhibition is caused by osmotic effects. In addition the most active compound present as an inhibitor was identified as coumarin.

Our thanks are due to Mr. N. Tadmor of the Department of Agriculture Research Station, for providing us with the large amounts of dispersal units of the two plants which were required for the investigations, as well as to Mr. M. Negbi, who assisted in preliminary selection of the seeds.

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The Stimulatory Effect of Light upon Growth and Carbon Dioxide Fixation in *Blastocladiella* III.

Further Studies, *in vivo* and *in vitro*

By

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Introduction

Blastocladiella emersonii (7) is a primitive water mold found in soils and bodies of fresh water. The colorless, vegetative plants of the fungus do not possess detectable carotenoids (5) and, of course, are devoid of chlorophyll. And yet, it grows, multiplies, and assimilates CO₂ more rapidly in the light than it does in the dark (5, 6). As far as we can tell, this places *Blastocladiella* in a unique position, perched as it were in the middle of the hazy limbo between the autotrophic and heterotrophic way of life.

A start has been made in relating light-stimulated development and the increased CO₂ fixation to associated changes in the metabolic machinery of the plant. *In vitro* studies with cell-free extracts and *in vivo* studies with submerged, liquid cultures involving several plant generations led to the conclusion (5) that the light-stimulated growth was due, at least in part, to a light-stimulated S.K.I. cycle; the latter involved a TPN-specific reductive carboxylation of ketoglutarate to isocitrate, cleavage of the isocitrate to succinate and a C₂-fragment, and the further TPN-specific reductive carboxylation of some of the succinate to ketoglutarate once again. It was suggested that

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the two-carbon compound produced with each turn of the cycle was involved somehow in the accelerated growth induced by light.

Subsequently, purely *in vivo* studies were made with surface cultures on agar media (peptone-yeast-glucose) on which plants were grown for one generation only (6). These investigations corroborated some of the conclusions obtained with submerged liquid cultures, and led to further, preliminary insight into the mechanism of light-stimulated growth at an organismal level of integration. In addition, it has been demonstrated (2) that the growth response to light also occurs on synthetic media (1) containing glucose, glutamate, methionine, thiamine, and inorganic salts.

We wish to report, now, upon some recent work which has been focused upon: (a), *in vivo* studies of the effects of environmental variables which limit or accelerate the stimulatory effect of light; and (b), further *in vitro* studies of the effects of light upon enzymatic reactions mediated by cell-free preparations of *Blastocladiella*.

Materials and Methods

In vivo studies. The strain of *B. emersonii*, the medium used (medium PYG, (1), a peptone, glucose, yeast extract medium solidified with agar), and the technique of following discharge of swarmers from individual, ordinary-colorless (OC) plants were those described by Cantino and Horenstein (6). Other specific details will be found in the figure legends and context.

In vitro studies. All *in vitro* studies were carried out at constant temperature (29°C). The intensity of white (fluorescent) light incident on the surface of reaction mixtures was 300 foot candles. Ferrocyanide assays were made by reacting ferrocyanide with ferric ion, the resulting ferrous ion being complexed with *o*-phenanthroline and the chelate thus formed measured at 510 m μ (modified from Krogman and Jagendorf 11). In all enzymatic runs, the crude enzyme was prepared as follows: washed, frozen mats of 3—6 day, light-grown, liquid cultures of OC plants in medium PYG were homogenized (8) at a level of one gm. wet wt./20 ml. 1/20 *M* phosphate buffer, pH 7.0. The homogenate was centrifuged (500 \times G for 5 minutes) and the supernatant dialyzed against distilled water at 5 C. for 17 hours. Dialyzed supernatants were then diluted three-fold with buffer and used directly as the source of enzyme. Other specific details will be found in the figure legends and context.

In Vivo Experiments

Light has a detectable two-fold effect upon the ontogeny of individual OC plants of *Blastocladiella* grown on the surface of solid media: (a), they reach maturity, form papillae, and discharge swarmers about three hours after similar plants incubated in the dark have done so; and (b), throughout ontogeny, light-grown plants are larger in size than those grown in the dark, the size

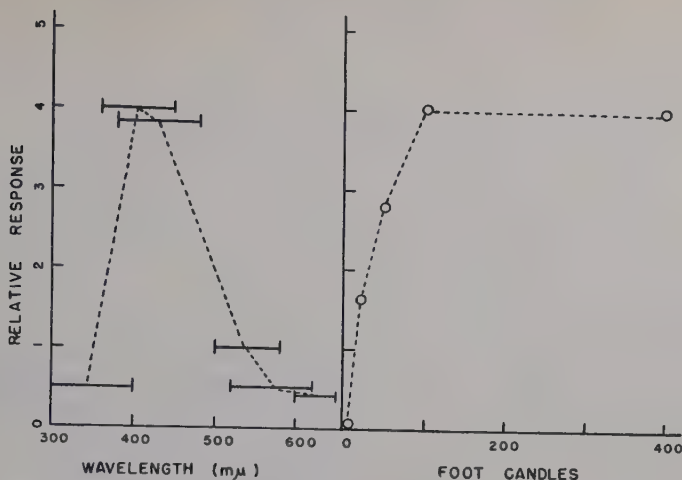


Figure 1, left. Rough action spectrum for light-stimulated growth of OC plants *Blastocycladiella* grown on solid medium PYG at 22 C. Relative response (arbitrary scale) derived from data on the percent of total plants discharged among populations exposed to different wave-length bands at the time that light-grown plants had just begun (e.g. under 5 % of total population) to discharge swarmers.

Figure 1, right. Relative response (as defined above) of OC plants on solid medium PYG at 22 C to different intensities of white (fluorescent) light. Plants were exposed throughout the full generation time.

difference becoming most striking towards the end of the generation time (6). As far as we have been able to determine, the increased size and the delayed discharge time are intrinsically related; thus, either parameter may be used to follow the stimulatory effect of light upon growth. In the studies reported below, the magnitude of the delayed discharge time of populations of individual, light-grown plants (relative to dark-grown plants) has been used as the criterion for establishing the effects of environmental factors upon the response of *Blastocycladiella* to light.

Effective wavelengths and optimum light intensities. Plants were exposed for their full generation time to various wavelength bands. While equal energy-levels were not used, total incident energies at the various wavelengths were of sufficiently-similar orders of magnitude to provide a reliable, preliminary action spectrum. The effective wavelengths for light-stimulated growth were in the blue end of the spectrum in the 400—500 mμ range. Wavelengths below 400 mμ and above 500 mμ neither inhibited nor accelerated the growth response (Figure 1, left). Between zero and 100 foot candles, the response curve for white light was roughly linear; above 100 foot candles, there was no further increase in growth and no inhibition (Figure 1, right).

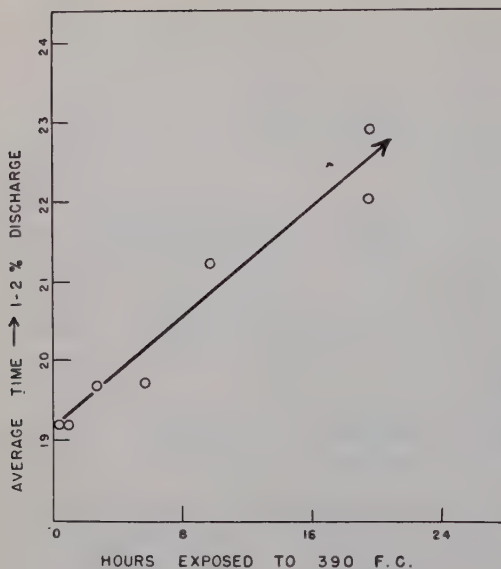


Figure 2. Relation between stimulatory effect of light on OC plants grown on medium PYG (measured as number of hours required for population to begin discharge, 1—2 %) and the duration of exposure of these populations to 390 foot candles of white light at 22°C.

Optimum duration of light exposure. Swarmers were allowed to germinate on plates of medium PYG; these populations were then placed under 390 foot candles of white light and subsequently transferred to darkness after various intervals, from zero time to ca 22 hours (the full generation time at 22°C). Between zero exposure (total darkness throughout ontogeny) and full exposure for the total generation time, the response curve was linear, with continuous light inducing maximum stimulation (Figure 2).

The effect of pH. Over the pH range 6 to 8, a level plateau was obtained for the response of populations incubated in total darkness (Figure 3). Using the counting techniques of Cantino and Hyatt (7), analyses revealed that viability of populations of swarmers germinated on medium PYG over this pH range was also constant (2). In the presence of light, however, the stimulatory effect of radiation upon growth increased with increasing pH levels (Figure 3). This was probably related, at least in part, to increased retention of atmospheric and metabolic CO_2 as the degree of alkalinity increased, and it demonstrated indirectly the vital role of CO_2 in this phenomenon (see below).

The effect of bicarbonate. The response to light was completely dependent upon the presence of bicarbonate or CO_2 (Figure 4). In the absence of added bicarbonate and atmospheric CO_2 , light had essentially no effect upon the growth and discharge time of the populations; in the presence of atmospheric CO_2 , light induced nearly-maximum effects. Above ca $5 \times 10^{-5} M$ bicarbonate, the response curve leveled off. At much higher concentrations of bicarbonate

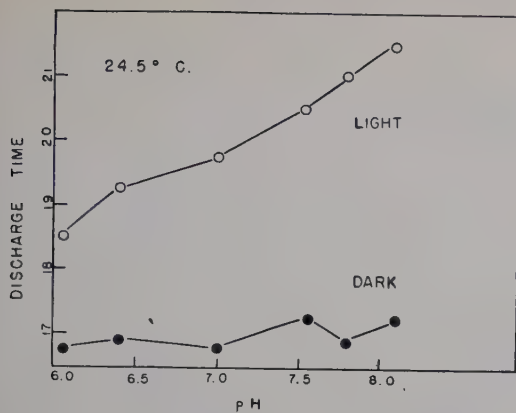


Figure 3.

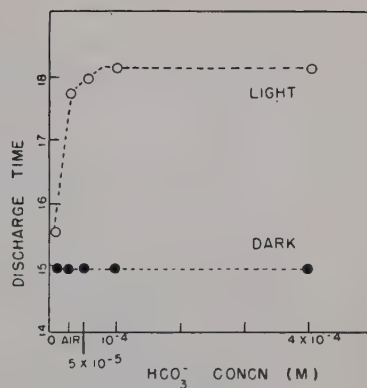


Figure 4.

Figure 3. Relation between stimulatory effect of light (390 foot candles) on OC plants (measured as discharge time; as defined in Figure 2), and the pH of medium PYG at 24.5°C. The effect of pH upon dark grown plants is shown for comparison. pH adjustments were made with KOH or HCl after sterilizing the medium.

Figure 4. Relation between the stimulatory effect of light (390 foot candles) on OC plants (measured as discharge time; as defined in Figure 2) and the bicarbonate content of medium PYG at 26°C. The medium was adjusted to pH 7.0 after sterilization, and different quantities of a 1/15 M phosphate—1/10 M bicarbonate solution of pH 7.1 were added simultaneously to provide the final concentrations shown. The CO₂-free cultures were prepared by inverting the petri dishes over a 10% KOH seal.

(e.g., approaching 10^{-2} M), growth rates decreased and the populations began to form RS as expected (3, 4, 9).

The effect of temperature. The effect of light was studied at 20, 22, 24, and 26°C. Increased growth rates at increased temperatures naturally caused a shift to the left (Figure 5) in the discharge curves for populations of OC plants, but it applied equally to both light and dark grown populations. Analyses of numerous replicate plates at all four temperatures revealed that the differences in the time at which light and dark grown populations begin to discharge is nearly a constant (Table 1). Thus, it appears as if the Q_{10} for light-stimulated growth is approximately unity, and thus may reflect some sort of limiting photochemical reaction.

The effect of glyoxylate and succinate. Our notions (5, 6) regarding the biochemical basis for light-stimulated growth suggested that: (a), reductive carboxylation of ketoglutarate gave rise, via isocitrate, to succinate and a C₂ fragment; (b), some of the succinate was further carboxylated to yield more ketoglutarate; and (c), the two-carbon compound and excess succinate prob-

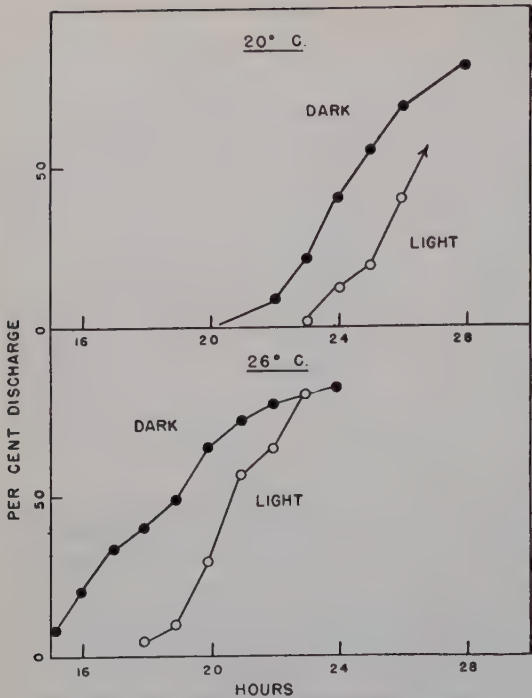


Figure 5. The rate at which populations discharged swarms when grown on medium PYG under white light (390 foot candles) and in darkness at 20 and 26°C. (cf. Cantino and Horenstein, 6, for methodology).

ably entered the metabolic machine of *Blastocladiella* in such a way as to yield the light-induced increase in growth. Recent studies by McCurdy and Cantino (4, 12) reveal the presence in *Blastocladiella* of an isocitritase which produces equimolar quantities of glyoxylate and succinate from isocitrate; on the other hand, citritase activity could not be detected. Thus, the C₂-fragment produced from isocitrate in the SKI cycle is probably glyoxylate, as had been suggested earlier on the basis of indirect evidence (5).

Since succinate and glyoxylate appeared to be the first cleavage products of the light-stimulated carboxylation of ketoglutarate to isocitrate, and the

Table 1. The effect of temperature upon the difference in time at which light-grown (390 foot candles) and dark-grown populations begin (e.g., ca. 1 % of the population) to discharge swarms.

Temperature, °C	Difference, in hours (average for three or more experiments)
20	3.0
22	3.0
24	3.5
26	3.5

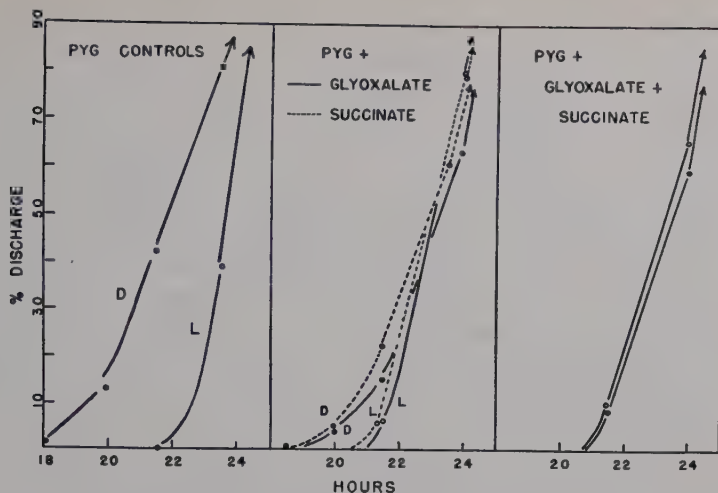


Figure 6. The effects of glyoxylate and succinate upon rates of discharge of populations grown on medium PYG under white light (390 foot candles) and in darkness at 23.0°C. Medium PYG was adjusted to pH 6.8 after sterilization, and glyoxylate and succinate (pH 6.8) were then added to yield 10^{-3} M final concentrations.

presumed precursors for the metabolic machinery involved in light stimulated growth, it was of interest to determine with our new assay procedure if they could be substituted for light. The results revealed that, indeed, they could (Figure 6). Succinate alone and glyoxylate alone were partially effective; but, the two together, in equimolar quantities, were able to substitute completely for light! The discharge curves for populations grown in the presence of glyoxylate and succinate in the dark were identical to those for control populations in the light (Figure 6), and the sizes of plants produced under these conditions were of the same order of magnitude as the controls grown with illumination.

These results are not only consistent with, but in fact provide significant support for our conclusions regarding the biochemical basis for the stimulatory effect of light upon growth and CO_2 fixation in *Blastocladiella* (4, 5, 6).

In Vitro Experiments

Previous experiments with whole cells and cell-free extracts provided a good deal of indirect and direct evidence that light-accelerated CO_2 fixation in *Blastocladiella* was mediated, at least in part, by a TPN-specific reductive carboxylation of ketoglutarate (5). In particular, experiments with crude,

cell-free extracts showed that light *decreased* the rate of oxidative decarboxylation of isocitrate to ketoglutarate via a TPN specific isocitric dehydrogenase, but that in the other direction, it *increased* the rate of reductive carboxylation of the ketoglutarate by way of the TPN specific dehydrogenase in the same cell-free preparation. However, it was impossible to ascertain with any degree of certainty if light: (a), produced additional reducing power to help drive the reductive carboxylation; (b), simply channeled, somehow, the pool of available reducing power towards this pathway, thus increasing the *efficiency* of its utilization without a real increase in net production of reduced nucleotide; (c), *directly* accelerated the reductive carboxylation of ketoglutarate in some mysterious fashion; or (d), *indirectly* accelerated reductive carboxylation of ketoglutarate by "pushing" reactions which led to production of ketoglutarate, or by "pulling" reactions sequential to the carboxylation of ketoglutarate (*e.g.*, cleavage of isocitrate, utilization of glyoxylate, etc.).

Consequently, we have been pursuing these *in vitro* studies further along two different directions: (a), the effect of light upon the cleavage of the isocitrate to succinate and glyoxylate, and the subsequent metabolism of glyoxylate, using purified enzyme preparations (McCurdy and Cantino, 12, to be the subject of a separate report); and (b), the effect of light upon the carboxylation of ketoglutarate and other enzyme reactions demonstrable in crude cell-free preparations. These latter studies, which provide corroborative evidence for the stimulatory effect of light on ketoglutarate metabolism and new evidence for the effect of light on other enzymatic processes, are summarized below.

In addition to most of the enzymatic activities usually associated with anaerobic conversion of glucose to lactic acid (4), low-speed ($500\times G$) supernatants of homogenates of OC plants of *Blastocladiella* also possess strong, TPN-specific, glucose-6-phosphate and 6-phosphogluconic dehydrogenases (Figure 8). Light has no detectable effect upon the course of these two reactions as measured directly by reduction of nucleotide at 340 m μ (Figure 8).

However, oxidation of these two substrates can be coupled to reduction of ferricyanide via carrier quantities of nucleotide. The reduced nucleotide produced reacts non-enzymatically with the ferricyanide to form ferrocyanide, the latter can be measured as the ferrous-*o*-phenanthroline complex (11), and the course of the reaction followed at 510 m μ . Under these conditions, light does have a marked effect (Figure 9); it apparently causes an abrupt cessation of the reduction of ferricyanide to ferrocyanide. The effect of light can be followed more easily and more directly, but with less sensitivity, by way of the rate of disappearance of the ferricyanide peak at 423 m μ (Figure 10). These response curves are corrected for all controls; *e.g.*, the low endogenous

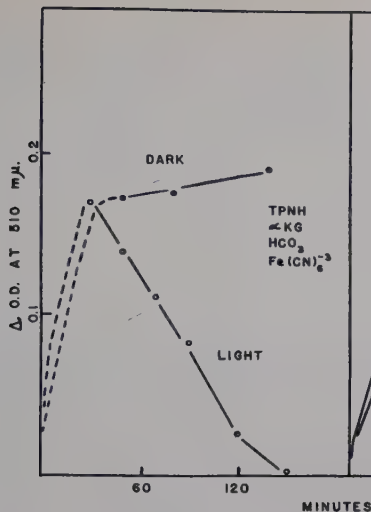


Figure 7.

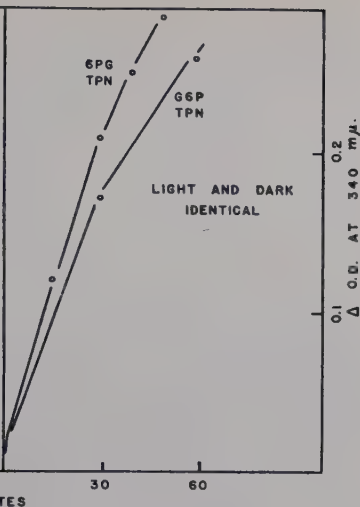


Figure 8.

Figure 7. Time course for the disappearance of ferrocyanide (in presence and absence of light) in reaction mixtures containing 1.6 ml. enzyme in total volume of 8.0 ml., pH 7.0, with following ingredients at concentrations indicated; reduced triphosphopyridine nucleotide (TPNH), 10^{-5} M; α -ketoglutarate (aKG), 10^{-3} M; bicarbonate, 10^{-3} M; ferricyanide, 5×10^{-4} M; and phosphate buffer, 10^{-2} M. Suitable aliquots were removed for analysis as the reaction progressed.

Figure 8. Time course for the oxidation of glucose-6-phosphate (G6P) and 6-phosphogluconic acid (6PG) in light and dark (as measured by reduction of triphosphopyridine nucleotide (TPN) at 340 mμ) in reaction mixtures containing 1.6 ml. enzyme in total volume of 8.0 ml., pH 7.0, with following ingredients at concentrations indicated: TPN, 10^{-4} M; glucose-6-phosphate or 6-phosphogluconic acid, 7.5×10^{-4} M; MgCl_2 , 10^{-6} M; KCN, 10^{-4} M; and phosphate buffer, 10^{-2} M.

levels without hexose phosphate, without nucleotide, with DPN instead of TPN, with boiled enzyme, etc. Furthermore, rates for endogenous oxidation of reduced TPN in the absence of other substrates by these cell free systems are identical in the light and in the dark. In addition, the rates for non-enzymatic reduction of ferricyanide by reduced TPN are also essentially the same in light and dark.

Thus, the only logical conclusion that occurs to us is that light mediates the selective utilization of reduced TPN by some product (or products) of the oxidation of glucose-6-phosphate beyond the 6-phosphogluconic acid stage, in such a way as to effectively shunt the reduced TPN away from, or prevent it from reaching, the site of its reaction with ferricyanide.

Because it had been established that light accelerated reductive carboxylation of ketoglutarate via reduced TPN (5) these new observations provided

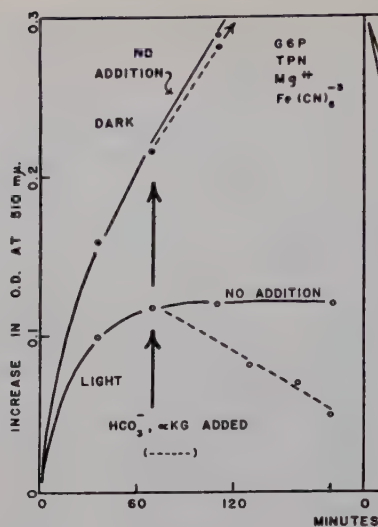


Figure 9.

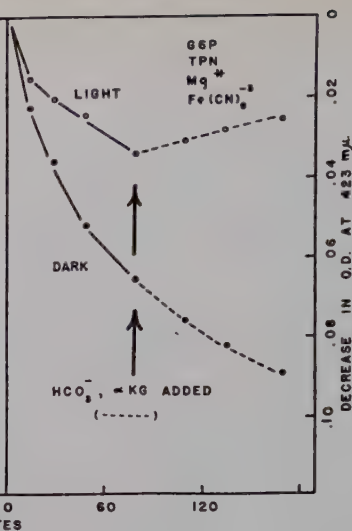


Figure 10.

Figures 9 and 10. Time course for the production of ferrocyanide and the disappearance of ferricyanide, respectively (in light and dark) in reaction mixtures containing 1.6 ml. of enzyme in total volume of 8 ml., pH 7.0, with following ingredients at concentrations indicated: TPN, 10^{-4} M; glucose-6-phosphate, 7.5×10^{-4} M; MgCl, 10^{-6} M; ferricyanide, 5×10^{-4} M; and phosphate buffer, 10^{-2} M. Ketoglutarate and bicarbonate, when added, each 10^{-3} M.

an opportunity to check this conclusion once again by yet another procedure. Ketoglutarate and bicarbonate were added to the ferricyanide-hexose phosphate-TPN incubation mixtures after the reaction had progressed for some time (Figures 9, 10). The results showed a clear cut light effect upon the system, whether it was measured at 510 mμ (for ferrocyanide accumulation) or at 423 mμ (for ferricyanide disappearance). The curves suggested that ketoglutarate was being reduced more rapidly in the light than in the dark at the expense of the available reducing power (ferrocyanide via nucleotide carrier) present in the system. Direct analyses for disappearance of added ketoglutarate in similar reaction mixtures (by spectrophotometry of 2,4 dinitrophenylhydrazones derivatives; 10) revealed that it was disappearing more rapidly in the light than in the dark (Table 2), as did visual comparisons of chromatograms in which the disappearance of the free acid was followed with pH sprays. The effect of light upon the ketoglutarate carboxylation was just as striking (Figure 7) when reduced TPN was provided directly for reduction of ferricyanide (rather than depending upon its formation by oxidation of hexose-phosphate). After the initial surge in production of ferro-

Table 2. Disappearance of ketoglutarate in reaction mixtures containing ferricyanide, ketoglutarate, bicarbonate, TPN, glucose-6-phosphate (cf. legends for Figures 9, 10, but with ketoglutarate at 8 instead of 10 μ M/10 ml.) and incubated in light (300 foot candles) and dark. — μ mol. ketoglutarate/10 ml. reaction mixture.

Time, minutes	Light	Dark
0	8	8
25	5.5	7.1

cyanide via non enzymatic reduction of ferricyanide by the reduced TPN, ferrocyanide was reoxidized again in the light as ketoglutarate became reductively carboxylated; but, in the dark it did not do so. The same light effect was demonstrable starting with preformed ferrocyanide and carrier quantities of nucleotide to mediate electron transport to ketoglutarate.

The foregoing results corroborate earlier conclusions derived from studies with labeled and unlabeled substrates (5); namely, that in *Blastocladiella emersonii*, light accelerates reductive carboxylation of ketoglutarate via a TPN specific reaction, presumably by way of an isocitric dehydrogenase. However, in view of the obvious effect of light in the absence of added ketoglutarate and bicarbonate (Figures 9, 10), it now seems likely that a second, TPN-specific reduction of some component produced from the metabolism of 6-phosphogluconic acid is also accelerated by light.

Discussion

As a result of the discovery of light-stimulated growth in *Blastocladiella*, we have begun attempts to unravel the phenomenon at biochemical, biophysical, and organismal levels of integration.

The first approach made was biochemical and generally physiological in scope. The results obtained to date (5) have provided a working hypothesis for part of the mechanism by which light stimulates growth and, as a consequence, manifold avenues which require further exploration.

At the biophysical level, too, a beginning has been made; but here, an important and conspicuous barrier has yet to be overcome. It seems axiomatic that a colored light receptor must exist in *Blastocladiella*; a more detailed action spectrum should provide us with a reasonably-powerful tool in our search for it. But at the moment, we can only presume that the receptor absorbs in the 400 to 500 m μ range and suspect that it will turn out to be a yellowish substance. And yet, judging from all extractions made to date, the colorless plants of *Blastocladiella* which respond so strikingly to light appear

to be almost totally devoid of any colored materials. Being a fungus, *Blastocladiella* should not possess chlorophyll; we have actually looked for it, and as one might expect, we have found no trace of it. In addition, all attempts to detect colored carotenoids (by the extraction methods used to isolate small quantities, ca 0.01 $\mu\text{g}/\text{mg}$. dry weight from resistant sporangia, 5) have yielded negative results. The possibility that quinones, flavines or flavoproteins are involved is being pursued but, as yet, we have no definitive evidence for their possible role in this story. So far, only one colored substance has been found in extracts from very large quantities of OC plants; a slightly yellowish, 80 % alcohol-soluble fraction, separated by starch column chromatography, with an apparent absorption peak at 255 $\text{m}\mu$ but no well defined peaks in the 400 to 500 $\text{m}\mu$ range. Interestingly enough, it seems to be about twice as concentrated in light-grown plants as in dark-grown plants. But at the moment, the possible role of this substance in light-stimulated growth remains a moot point.

And finally, there is the organismal level of integration. In the last analysis, our interest lies in the organism, not in the taxonomy of its enzymes. Our newly-developed assay procedure for the effect of light upon single generations of OC plants of *Blastocladiella* has provided us with new vistas which are now ripe for dissection and analysis. Many questions previously impossible to attack with submerged liquid cultures involving many generations can be approached, now, in more definitive fashion. Some of the implications arising from in vitro studies with dissected pieces of biochemical machinery and the S.K.I. cycle can be put to a partial test at an in vivo level. Notwithstanding the permeability problem, presumed key intermediates arising from the effect of light upon crucial enzyme systems can be tested for their effectiveness as substitutes for light, and for their effect upon the biological machine. And finally, the effect of light at a biophysical level can be approached directly and under more precisely-controlled conditions. The areas already attacked which deal with the quantity and duration of light required, the effects of temperature thereon, the biophysical nature of the limiting light reaction, all can be pursued further and hopefully brought to bear upon a unique phenomenon; that of an aquatic mold which, though lacking chlorophyll, grows more rapidly and fixes more CO_2 in the light than it does in the dark.

Summary

At an in vivo level, thin-walled, ordinary colorless plants of *B. emersonii* increase more rapidly in size and have a longer generation time in the light than in the dark. Maximum stimulation occurs with continuous exposure to

100 foot candles of white light. The effective wave lengths lie in the 400 to 500 m μ range. Atmospheric CO₂ or 10⁻⁵ M bicarbonate are essential; without them, light has no effect. Preliminary data for a six-degree temperature range suggest that the Q₁₀ for light-stimulated growth is unity. Finally, the effect of light can be duplicated by a mixture of glyoxylate and succinate, the presumed products of the reductive carboxylation of ketoglutarate which occurs in light-stimulated growth.

At an in-vitro level, the effect of light on ketoglutarate metabolism is corroborated. In addition, the data suggest that another, unidentified reaction involving TPN and a product of glucose-6-phosphate metabolism is also affected by light.

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The Sequestration of Calcium from Preparations of Wheat Coleoptile Cell Walls

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Introduction

The possibility that auxins might act as chelating agents appears to have been first suggested by F. Chodat in the discussion following a paper given by Thimann at the Botanical Congress in Stockholm in 1950. It is of some interest that copper was the metal specifically mentioned by Chodat as possibly involved in chelate formation. The first report that chelating agents may exhibit plant growth-regulating properties was made by Bennet-Clark (1956) at the Wye Symposium in 1955. EDTA at concentrations from 10^{-5} to 10^{-3} M was reported to stimulate extension growth of coleoptiles of wheat and oats and this activity was attributed to the chelation of calcium from the cell wall. A similar growth-regulating activity of EDTA¹ and other chelating agents was reported by Heath and Clark (1956) who claimed that EDTA at 10^{-5} M often increased the growth of wheat coleoptile segments as much as IAA at the same molar concentration. Generally, however, the activity of the chelating agents was less than that of IAA. Like Bennet-Clark they favoured a calcium-chelation hypothesis and cited the non-activity or somewhat inhibitory activity of non-chelating analogues of 8-hydroxyquinoline, which itself stimulates growth to a considerable extent. Heath and Clark (1956 b) suggested that highly active auxins such as IAA and 2,4-D might act as chelating or complex-forming agents. They claimed that IAA and EDTA, or

¹ EDTA = disodium ethylenediamine tetraacetic acid. 8-HQ = 8-hydroxyquinoline. IAA = indole-3-acetic acid. DIECA = diethyl dithiocarbamate.

IAA and 8-HQ exert some sort of mutually antagonistic effect on root growth. Burström and Tullin (1957) have failed to substantiate these claims and find that cell elongation of the roots of intact wheat seedlings is not affected by EDTA at all, although cell division is adversely affected. On the other hand, IAA inhibits elongation of root cells but not cell division.

Weinstein *et al.* (1956 a; 1956 b) claim that EDTA stimulates the growth of segments of hypocotyls of etiolated lupin seedlings and of whole sunflower plants, and elicits epinastic responses in soybean leaves. They favour an explanation based on chelation of the calcium of the cell wall and hold this to be supported by the activity of uncomplexed EDTA but not of the iron chelate. On the other hand, Fawcett, Wain and Wightman (1956) find that DIECA and EDTA are much less active on wheat coleoptile segments than was reported by Heath and Clark (1956 a) and that EDTA is completely inactive in the tomato leaf epinasty and pea curvature tests. They attribute such low activity as that shown in the wheat coleoptile test to changes caused by sub-acute toxicity and state that the activity appears only "at the near toxic range, at a concentration of 10^{-3} for EDTA".

The paper of Heath and Clark (1956 a) came to our notice just after our investigations were begun and most of the other work has appeared since then. Our attention had been drawn to the possible effects of chelating agents on growth by observations (Carr and Ross, unpublished) of the morphogenetic effects of "Zineb" a commercial fungicide, zinc bis-diethyldithiocarbamate, on leaves of *Xanthium pensylvanicum*, and by the results of attempts (Ng, unpublished) to utilise copper chelating compounds in the striking of cuttings of "difficult" tree species (*e.g.* Eucalyptus spp.). The experiments to be reported in this paper were designed to test the hypothesis that the growth-promoting effects of EDTA on coleoptile segments of wheat (which in our experiments were not as large as those found by Heath and Clark) are due to sequestration of calcium from the cell wall. We considered it essential to buffer the solutions because chelate-formation depends on pH. For a variety of reasons, citric-phosphate buffers (modified McIlvaine's buffers: 0.2 M K_2HPO_4 , 0.1 M citric acid) were used. These buffers cover a wide range of pH. Potassium (Cooil 1952, Thimann and Schneider 1938) and citric acid are said to enhance auxin-induced growth (Thimann and Bonner, W. D. jr. 1948). It became clear at an early stage that these buffers at 0.015 M and pH 4 to 5.5 are in fact much more active in complexing calcium than the chelating agents to which so much attention has been given in the literature. Citric-phosphate buffers have been widely used in adjusting the pH of solutions for growth tests (*e.g.* Bonner 1934, Leopold and Guernsey 1954) but as far as we can discover no reference has been made to their effect on the calcium of the cell wall.

The experiments to be described in this paper were undertaken to determine the amount of calcium in the cell walls of coleoptile segments such as are used in growth experiments, and to test by direct analysis whether the chelating agent, EDTA, at physiological concentrations and pH values can affect the content of calcium in the cell wall. As far as we are able to ascertain, no determinations of the amount of calcium in the cell walls of coleoptiles have been published. Kögl and Mulder (1956) have published a very inadequate account of some work on the chemical constituents, including calcium and pectin, of the cell walls of shoots and roots of 3-day-old oat seedlings, but the amounts are given only as percentages of control plants (not treated with auxin).

Materials and Methods

Wheat coleoptiles (variety "Gabo") were obtained in the following manner. Wheat grains were soaked in tap water in a dish for 15–20 hours overnight at room temperature (18–20°C). After soaking, the grains were placed in parallel rows on glass plates covered with wet filter paper, with the groove side of each grain next to the paper and the plumule pointing upwards. The glass plates were stood at an angle of 60° in glass museum jars with water added to the bottom of each jar. This procedure gives straight coleoptiles in normal orientation. The museum jars were placed in a chamber (that of Avery *et al.* 1939) in which the temperature was 25–26°C and humidity 90–100 %. The plants were allowed to grow for 92 hours after the beginning of soaking. Each selected coleoptile was then severed from its mesocotyl and, after discarding a 3 mm. tip, a segment approximately 10 mm. long was cut from it. The segments were slit and the leaves extracted and discarded.

Calcium was determined by two methods, flame photometry and iodometry. For flame photometry an EEL photometer was used at full sensitivity. Standard solutions of CaCl_2 were made up from analar chemicals, adjusted to contain 1 % HCl, and used for calibration. The calibration curves were reproducible and linear. The titration (iodometric) method due to Siwe (1935) and described by Glick (1949) was used. This depends on conversion of the calcium to the oxalate. The washed oxalate is dissolved in nitric acid and the liberated oxalic acid titrated with 1/100 *N* permanganate until the latter is in excess. The excess is then determined by iodometry, using 1/100 *N* thiosulphate. According to Glick the method, carried out as a micro-analysis, will determine 5 µg. of Ca with an accuracy of 3.5 %, but in the estimations of cell wall calcium the determinations were always too high as compared with those made by the flame photometer. We have considered it valid to correct them to bring them in line with the photometric determinations. On the other hand the titrimetric method was a useful check because certain treatments resulted in anomalous high readings in flame photometer determinations. These anomalies may have been due to interference by sodium. In certain cases, therefore, the corrected titrimetric readings have been taken as the only valid determinations. For the titrations an "Aglā" micrometer syringe was used as a microburette.

Results

Experiment 1. Effect of citric-phosphate buffer (pH 5.28) and buffered EDTA on the calcium content of coleoptile cell walls.

An outline of the method of preparation of the cell wall material and the procedure of this experiment is given below:

Coleoptile segments homogenized in H ₂ O, centrifuged, re-suspended and centrifuged 5 times.		
Residue		Supernatant: discard.
Re-suspend in H ₂ O		
Aliquot	Aliquot	Aliquot
Suspend in EDTA 200 mg/l buffered to pH 5.28, 24 hrs. 25°C in the dark	Suspend in buffer pH 5.28, 24 hrs. 25°C in the dark.	Dried at 95°C. Dry weight determination.
Centrifuge, wash and cen- trifuge 5 times with H ₂ O. Dry residues at 95°C.		
Dry wt. of residue	Dry wt. of residue	
Ash in muffle furnace, di- gest ash with HCl. Make up to 10 ml. with distilled wa- ter. Determine Ca by flame photometry.		

The coleoptile segments (nearly 9 gm.) were homogenized in glass distilled water in a Wareing blender, the homogenate was centrifuged and the supernatant discarded. This washing procedure was repeated 5 times in all, and the final residue was re-suspended in distilled water and 5 ml. aliquots taken. Two treatments were applied. One was to add, to a 5 ml. aliquot, 5 ml. of buffered EDTA, 200 mg/l, pH 5.28. The tube containing this mixture was shaken on a horizontal shaker for 24 hours at 25°C. In the other treatment 5 ml. of buffer alone was added to the aliquot of cell wall material. This also was left on the shaker for 24 hours. The residues were then collected, washed 5 times, dried in an oven for 24 hours then

Table 1.

Total fresh weight of coleoptiles		996.3 mg.
" dry " " "		36.0 mg.
Dry wt. as percent. of fresh weight		3.6 %
Treatment	Mean dry wt. of cell wall material g.	% Ca in dry wt.
EDTA in buffer	0.03313	0.098
Buffer alone	0.03330	0.027

Table 2.

Treatment	Dry wt. gm.	Ca content μ g.	% Ca in dry wt.
Control	3.3366	19,150	0.115
Citric-Phosphate buffer at pH 5 ...	1.1456	1,520	0.0265
EDTA (200 mg/l) in buffer at pH 5	0.9948	6,860	0.138

ashed in a muffle furnace (450°C) overnight. The ash was cooled and taken up in 1 ml. of 10 % HCl by heating on a hot plate. The volume was made up to 10 ml. with glass distilled water, and the calcium in the resulting solution determined by flame photometry in the usual manner.

The results are given in Table 1. The content of calcium is expressed as a percentage of the dry weight of the cell wall. These results will be discussed together with those of experiments 2 and 3.

Experiment 2. Effect of citric-phosphate buffer and buffered EDTA on calcium content of filter paper.

It seemed likely from the results of experiment 1 that the buffer alone had considerably reduced the calcium content of the wall material. This might be expected because at pH 5 the citric-phosphate buffer acts mainly as citric acid which at this pH is an efficient complexing agent for calcium. Actually the "buffer alone" treatment had been included as the "control" and the calcium complexing action of the buffer was realised only when it became clear from further determinations that the calcium content of the coleoptile cell wall is normally about 0.1 %. We decided to see whether the buffer would remove calcium from "ashless" filter paper which contains amounts of calcium comparable with those in cell wall preparations.

Six ashless filter papers (Whatman No. 41) were homogenized and the slurry filtered and washed 5 times, each time with 150 ml. of double-glass-distilled water. The debris was then dried and weighed. Two filter papers were similarly homogenized and washed for each of the experimental treatments. The material from two papers was suspended for 8 hours in 100 ml. of buffer at pH 5. The material from two more filter papers was treated similarly with 100 ml. of EDTA (200 gm./l.) made up in buffer at pH 5. In each case the buffer concentration was 0.015 *M*. After treatment the debris was collected by filtration, washed and centrifuged twice and then oven dried. The oven-dry material was ashed and the ash taken up in HCl. Aliquots were taken for calcium determination by iodometry. The results are given in Table 2.

It is clear that the paper treated with buffer lost a great deal of its calcium whereas the paper treated with EDTA in buffer did not lose calcium. (The difference between "EDTA-treated" and "control" is probably within the sampling and other errors of the methods.) We cannot put forward a satisfactory explanation of the "protective" nature of the interaction between

Table 3.

Treatment	Dry wt. gm.	% Ca by photometry	% Ca by iodometry	Mean % Ca
Control (A)	0.0291	0.1457	0.1267	0.1373 (by photometry)
„ (B)	0.0323	0.127	0.1478	
„ (C)	0.0260	0.14	(not done)	
pH 5	0.0162	—	0.0285	
pH 7	0.0151	—	0.11	
EDTA	0.0253	—	0.098	

Mean of all samples 0.1022 %.

EDTA and citric-phosphate buffer, but it is possible that such an interaction may explain the lack of effectiveness of the EDTA-treatment in Expt. 1 compared with buffer alone.

Experiment 3. Treatments applied to coleoptile segments prior to homogenization.

Because of the tedium of preparing large quantities of coleoptile material and to minimise losses in homogenization, each sub-sample of coleoptiles in this experiment was ground separately in a glass Potter-Elvehjem homogeniser, using an up-and-down motion. Each suspension thus obtained was transferred to a centrifuge tube in which the material stayed during all subsequent treatments.

Six samples of coleoptiles were used. Three (A, B, and C) were homogenized immediately. The other three lots were floated for 3 hours before homogenization on solutions either of citric-phosphate buffer (0.015 *M*) at pH 5 or pH 7, or of EDTA (10^{-5} *M*) adjusted to pH 5 with dilute HCl and dilute NaOH. These treatments were to simulate the conditions under which the buffer at these pH values has been used in growth experiments (Bonner 1934) and to compare the effects on cell-wall calcium with those due to EDTA at a concentration and pH which has been found by experiment to be near the optimal for growth stimulation of wheat coleoptiles. Each sample was finally homogenized and alternately washed and centrifuged five times using cold distilled water.

Each residue was treated with 5 ml. of strong HCl (1 : 1) for 12 hours. The cell debris had then become rather voluminous probably owing to swelling of pectic materials. The HCl extract was centrifuged and aliquots taken and diluted for calcium estimation. The control samples A and B gave consistent readings with both photometric and titrimetric methods, but the treated samples gave abnormally high values with the photometric method. This was provisionally attributed to interference either by potassium or by sodium. Values for calcium in the treated samples are therefore those found by the titrimetric method. The results are given in Table 3.

Discussion

According to Thimann and Bonner (1933) the amount of pectin in cell wall material of oat coleoptiles is about 8 % of the dry weight. Assuming all

the pectin to be in the form of pectic acid (which is extremely unlikely) and this to be fully saturated with calcium, the maximum amount of calcium (as Ca-pectate) in the dry weight of the cell wall may be calculated to be 0.8 %. Calcium is known to constitute about 0.12 % of the dry weight of the plumule of wheat seedlings (Czapek 1925, similar figures are quoted from Burström in Lundegardh 1951). It seems probable, however, that much of this calcium is in the protoplasts. However, a danger exists in homogenizing plant material for the purpose of extracting cell wall materials that some of the calcium from the protoplast and vacuole may become bound to the cell wall. Indeed, during the series of experiments outlined above a sample of cell wall material of wheat coleoptiles was found to contain, after the first washing with 150 ml. of water, 0.205 % of calcium, whereas a sample of the same material after the fifth washing contained only 0.09 % calcium. It is of course impossible to say whether the successive washings remove calcium which formed part of the cell wall in the natural state of the cell or whether it is merely extraneous calcium derived from the protoplasm and vacuole which is thus removed. It seems reasonable to assume that calcium readily removed by washing with cold water was not bound to the cell wall in its natural state. For the rest, it may be assumed that the remaining non-uronate calcium forms part of the protoplasmic structures in the cell wall. This calcium is unlikely to affect directly the plasticity of the cell wall. For our purposes, the calcium content of wheat coleoptile cell wall preparations is about 0.1 % of the dry weight of the cell wall. Some of this may be bound, of course to uronic acids forming part of the hemicellulose of the cell wall, although little is known of the affinity of the native hemicelluloses for calcium. In any case, the amount of cell wall calcium is far below the highly theoretical maximum quoted above (0.8 %), based solely on expected content of pectic compound. Now, if the hypothesis which explains growth promotion by chelating compounds on the basis of sequestration of calcium is valid, then one might suppose that it should be possible to demonstrate a reduction in the calcium content of the cell wall following treatment with substances such as EDTA at physiological levels of concentration and pH. It will be shown in a subsequent paper that EDTA exerts its growth-promoting effects on wheat coleoptiles in the pH range 4 to 6. In fact, as may be seen from the data there is no evidence from our experiments that EDTA at pH 5 can effectively reduce the calcium content either of cell wall material or even of filter paper. However, citric-phosphate buffer at pH 5 effectively removes calcium from cell wall material. Why, then, is calcium not removed from the wall by the same buffer (at the same pH) in the presence of EDTA? We can provide no convincing answer to this question. It hardly seems probable that the dissociation of EDTA, suppressing that of citric acid, could be responsible since

the concentration of EDTA is so low (200 mg./l.). Moreover tests with buffer and EDTA acting together on suspensions of calcium sulphate do not show the kind of antagonism which appears when the calcium is attached to or forms part of filter paper or the cell wall. The effect may depend on adsorption, or on the fairly strong positive charge which is a feature of these cellulosic materials. EDTA may be selectively adsorbed by these materials and prevent the calcium from being complexed by the citric acid simply by masking it, without actually chelating it. However, if EDTA does not reduce the calcium content of the cell wall at pH 5 any explanation of its effects on growth which depends on the sequestration of calcium from the cell wall is patently untenable.

In order to avoid the difficulties which are involved in the use of fresh cell wall material owing to contamination with cytoplasmic constituents, some of our experiments have been performed with filter paper. It occurred to us, for instance, that carboxylic groups freed of calcium might readily pick up calcium liberated from the protoplasm or vacuole, and this might make it difficult to detect changes in cell wall calcium brought about by treatments applied prior to homogenization. However, it is clear from the results of experiment 3 that interference of this kind must be very small. Treatment of fresh coleoptiles with buffer alone at pH 5 resulted in a decrease in cell wall calcium of the same order as that achieved by treatment of washed cell wall material with the same buffer. On the other hand, fresh coleoptile segments treated with EDTA adjusted to pH 5, or with buffer at pH 7 did not lose significant amounts of calcium.

It is rather surprising that proponents of the "calcium chelation hypothesis" have overlooked the well-known calcium complexing properties of citric acid (*e.g.* Gomori 1946). There is no reason to doubt that calcium plays an important role in antagonising the plasticization of the cell walls of coleoptiles (*e.g.* Cooil and Bonner 1957) and that removal of calcium may facilitate extension growth. On these grounds alone one could expect that citric-phosphate buffer at pH 4—5 (at which the calcium-complexing activity is at a maximum) should promote extension growth in coleoptiles. Indeed it has been known ever since the classical work of Bonner (1934) that this is the case, although the interpretation given by its author was based on the assumption that it is the undissociated molecules of the weakly acid "growth substance" which alone are active, and that the buffer caused a shift in the internal pH to about pH 4.8, thus reducing the dissociation of the "growth substance" to a level which permitted maximum growth.

In point of fact, however, the material of Bonner's experiments consisted of segments of coleoptiles the tips of which had been removed some two hours previously. It was supposed by Bonner that they contained some "growth

substance" and that the dissociation of this endogenous auxin was suppressed by the buffer. It is now believed, however, that the internal level of free auxin in such segments is very low indeed. Even a brief immersion (one hour) in buffer at pH 4.1 considerably increased the plasticity of the cell walls and the growth rate, as compared with immersion at pH 7.2. On the basis of the results of Experiment 3 of this paper, it seems inherently probable that even during a brief immersion in buffer at pH 4.1 an appreciable fraction of the cell wall calcium would be removed. The resultant increase in plasticity may reduce the wall pressure and enable growth to take place. In fact Bonner states that "his experiments demonstrate clearly that . . . the difference in the action of pH 4.1 and pH 7.2 is accompanied by a considerable difference in the plasticity of the cell wall".

Many authors (*e.g.* Brecht 1936) have attempted to explain Bonner's results in various ways, but none seems to have adopted the explanation which now seems obvious. Söding (1952) has discussed a number of the hypotheses alternative to that of Bonner and has come to the conclusion that "the whole problem is apparently still unexplained". Controversy still exists on the role of dissociation in the activity of auxin. Thimann and Schneider (1938) in a critical discussion have concluded that "in the absence of buffer, pH exerts no influence on the response to auxin". The main influence of dissociation is on the entry of externally applied auxin into the cell, in which case "the auxin salt apparently enters the cell less rapidly than the acid". Nevertheless Went and Thimann (1937) still maintained that "the action of acid is through its effect on the auxin in the plant" in agreement with Bonner (1934).

The remote possibility has recently been raised that citric-phosphate buffers might stimulate the growth of coleoptiles because of the chelation of manganese, thus decreasing the activity of indole-acetic oxidase (Stutz 1957). However, Stutz has shown that the pH optimum of this enzyme (obtained from lupins) is about 6.3—6.5 and if the buffer were to stimulate growth by inhibiting the IAA-oxidase system it would be reasonable to expect it to do so in this pH range rather than at pH 4—5. Moreover, orthophosphate (as used in McIlvaine's buffers) is less inhibitory than pyrophosphate, and citric acid is inhibitory only when manganese is added to the reaction mixtures. An explanation of the stimulatory action of citric-phosphate buffer on the growth of intact coleoptiles in terms of the complexing of calcium therefore fits the facts more closely than one which might be based on the inactivation of IAA-oxidase.

No objections can be raised to the use of buffers in growth experiments with coleoptiles so long as it is realised that in certain pH ranges the buffer alone may have stimulatory effects. Indeed it is clearly essential to standardise the conditions under which experiments are performed as closely as

possible, as Bonner and Foster (1955) have pointed out. Certain buffers other than McIlvaine's should be recognized as capable of acting in the same manner as citric-phosphate buffers at pH 4—5. For instance, the potassium maleate buffer of Bonner and Foster (*loc. cit.*) may remove calcium from the cell wall. Several of the organic acids, including maleic acid, form complexes with calcium.

Summary

The calcium content of cell wall preparations of wheat coleoptile segments has been determined. It is about 0.1 % of the dry weight. The calcium content of cell wall preparations is considerably reduced by treatment with citric-phosphate buffer at pH 4—5 but not by solutions of EDTA at about pH 5.

When coleoptiles are floated on buffer solutions for 3 hours prior to homogenization the content of calcium in the cell wall is reduced by the buffer at pH 5 but not at pH 7. A similar treatment with EDTA adjusted to pH 5 does not reduce the content of calcium in the cell walls.

These results are discussed in relation to the hypothesis that EDTA (and similar chelating agents) stimulate extension growth by chelating calcium from the cell walls, thus increasing their plasticity. Older explanations of the stimulatory effects of citric-phosphate buffer at pH 4—5 on coleoptile growth are rejected in favour of an explanation based on the ability of this buffer to complex calcium from the cell wall.

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Effects of pH on the Activity of Chelating Agents and Auxins in Cell Extension

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Introduction

In the first paper of this series (Carr and Ng 1959) previously published work on the growth-promoting activities of chelating compounds was summarised. The hypothesis which has generally been adopted (*e.g.* Tagawa and Bonner 1957) is that originally proposed by Bennet-Clark, namely the sequestration of calcium from the cell wall. In this paper we present experiments carried out to test this hypothesis by exploring the effects of chelating agents on growth in relation to pH. Apart from the quite detailed study by Burström and Tullin (1957) of the effects of EDTA, calcium, manganese and iron on the growth of roots of intact wheat seedlings in nutrient solutions at pH 7, there has been little really critical work on this topic. In few or none of the published experiments in which DIECA, EDTA or other chelating compounds were found to stimulate growth of coleoptiles were the solutions buffered. This omission is a grave one because the ability to form chelates with metallic ions is a function of the pH. Moreover the importance of pH in comparative studies of the biological activity of weak acids and bases has been stressed by Simon and Beevers (1952). In comparing the activities of unbuffered solutions the pH of DIECA (about 8) would be quite different from, say, that of di-sodium EDTA (about 4).

Materials and Methods

Coleoptiles of wheat and oats were grown by the methods described in the previous paper. Freshly harvested "Gabo" wheat was used in the experiments. Oats

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(variety "Algerian") were husked and were grown for 69—72 hours. One day after planting, the germinating seeds were exposed to red light for 3—4 hours to suppress mesocotyl elongation. This treatment was not applied to wheat, which does not require it.

All subsequent operations were carried out in a room kept at constant temperature (25°C) and ambient humidity. Discarding a 3 mm. tip, a 10 mm. section was cut from each selected coleoptile, using a microtome like that described by Nitsch and Nitsch (1956). Coleoptiles were selected for uniformity, and only one segment was cut from each, leaving the first leaf inside. This tends to decrease the variability within occasions, simplifies the procedure and eliminates injury due to handling. Moreover, Bentley and Housley (1954) and Hancock and Barlow (1952) have found that growth is better if the primary leaf is left inside the segment.

Ten segments, randomly chosen, were placed into 10 ml. of test solution contained in a 150-ml. Erlenmeyer flask. These flasks were gently shaken on a horizontal shaker, following the recommendation of Nitsch and Nitsch (1956), for 25 hours in the dark, at a temperature of 25°C. The lengths of the segments were then measured to the nearest 0.02 mm. using a vernier travelling microscope.

Solutions were made up in glass distilled water. 40 mg. of indole-3-acetic acid (Analar grade, L. Light Pty., Sydney) was dissolved in 1 ml. of 95 % ethyl alcohol and this made up to 100 ml. with water. Serial dilutions were made from this stock. B.D.H. Analar grade sodium-DIECA, disodium-EDTA, and 8-HQ were used. A sample of the compound *s*-carboxymethyl dimethyldithiocarbamate (DTC) was kindly presented to us by Prof. R. L. Wain, Wye College, Kent. A fresh lot of each solution was made up on the day previous to each experiment and kept in the cold overnight. Except where stated, all the solutions and control media were buffered with a modified McIlvaine's buffer (0.2 M K_2HPO_4 , 0.1 M Citric acid) at a final concentration of 0.015 M.

The measurements were subjected to statistical analysis using the *t*-test, and adopting the 5 % level of significance. For graphical comparisons the data were also expressed as percentages of the lengths attained by segments grown in control media. The number of experiments carried out was too large to permit of the publication of any but representative data. The rest are available for examination in the library of the Botany Department, Melbourne University (Ng 1958).

Experimental. 1. DIECA

DIECA is extremely unstable in acid solutions above 20°C (James and Garton 1952). It is therefore difficult to test its effects on growth at any pH value below 7. In one experiment in which oat coleoptile segments were used and the solutions were not buffered, DIECA at 0.02 mg./l. gave a significant stimulation of growth (Figure 2) but when buffered to pH 5 (Figure 1) there was no indication of any stimulation of the growth of either oat or wheat coleoptiles and some concentrations were significantly inhibitory to oat coleoptiles. With wheat coleoptiles DIECA had no activity at pH 7 (Figure 3), but at pH 8, 2 mg./l. was stimulatory and higher concentrations up to 200 mg./l. were significantly inhibitory. These conclusions are based on the

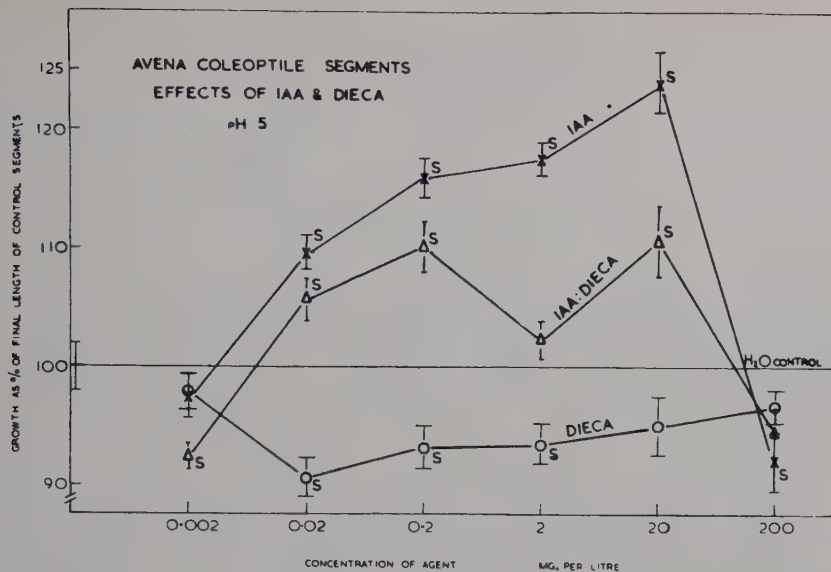


Figure 1.

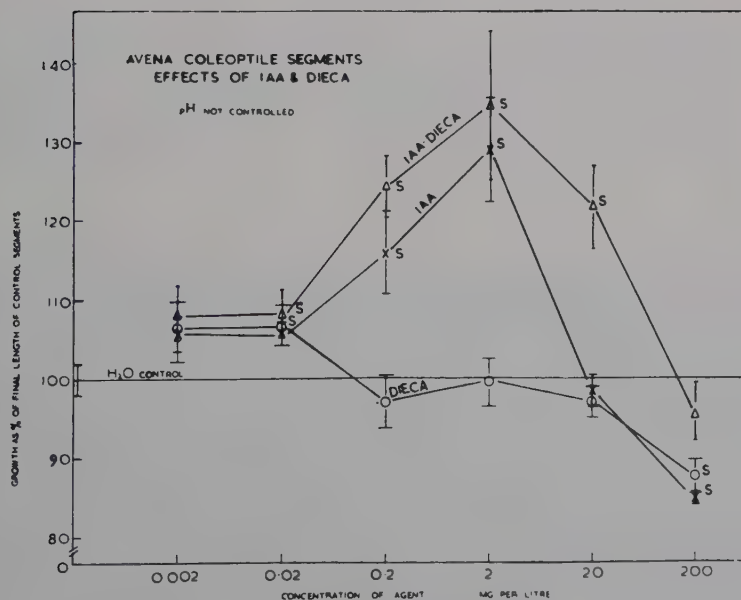


Figure 2.

Figures 1, 2 and 3. Effects of different concentrations of DIECA and IAA on extension growth of coleoptile segments in relation to pH. Figure 1 (oat coleoptiles), pH 5 (means of 3 experiments). Figure 2 (oat coleoptiles), unbuffered (one experiment). Figure 3 (wheat coleoptiles) pH 7 (2 experiments). Graphs show effects

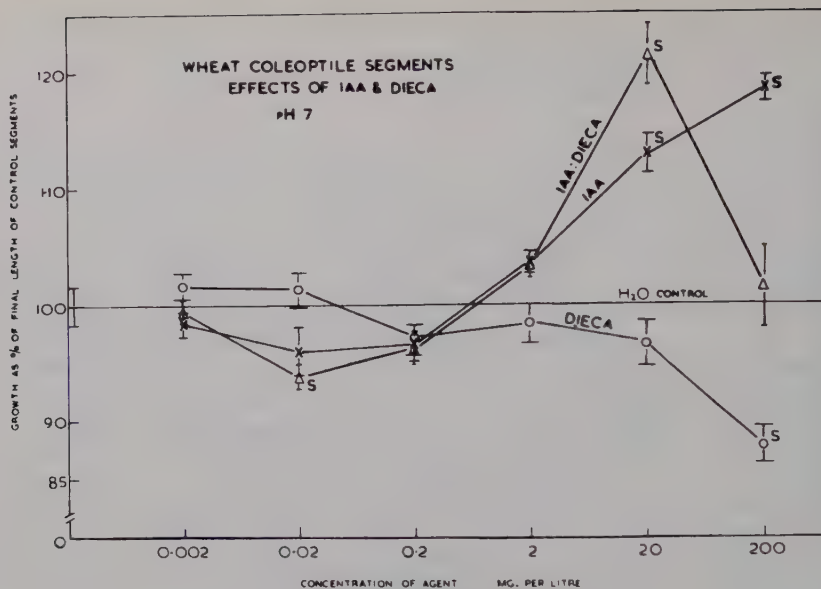


Figure 3.

of IAA alone, DIECA alone and IAA : DIECA, 1 : 1, each agent at the concentrations (mg./l.) shown on the abscissa. Ordinates, growth as percentage of final length of control segments in buffer (distilled water in Figure 2). Note: in these and the other figures vertical lines through the points denote the standard error for each point. Points marked "S" are significantly different from the controls at the 5 % level ("t" test).

results of nine experiments. Heath and Clark (1956 a) claim that although the effects of IAA and EDTA acting separately are practically identical evidence of a mutual antagonism between them exists in that inhibition due to either substance at a concentration of 10^{-5} M is completely or almost completely reversed in the presence of the other agent at a concentration of 10^{-9} or 10^{-11} M. In a further paper they claim that a similar kind of mutual antagonism is demonstrable in the action of IAA and 8-HQ on the growth of roots. No mutual antagonism was displayed when 8-HQ was replaced by a non-chelating analogue, 2-HQ. A similar kind of antagonism is claimed for supra-optimal concentrations of IAA and 8-HQ acting on coleoptiles. Since IAA and the chelating agents interact in this way, they claim support for the concept that IAA itself acts as a chelating agent.

In the experiments with DIECA and IAA described below, each agent and mixtures of the agents in equal proportions were tested. In each case the concentration of each agent in the mixtures was the same as that of the agent

when used alone. Two experiments on oat coleoptiles are reported here. The results are shown in Figures 1 and 2. As already mentioned DIECA is unstable at pH 5 and the inhibition due to DIECA alone at most of the concentrations shown in Figure 1 may be attributed to the breakdown products. The mixtures of DIECA and IAA are far less stimulatory to growth than IAA alone, irrespective of concentration. In unbuffered solutions, on the other hand (Figure 2) the mixtures of IAA and DIECA are more effective at all concentrations than IAA. There is no evidence of antagonism. On the contrary DIECA and IAA appear to act synergistically. It should be emphasised however, that the pH values of different solutions (IAA, DIECA and IAA: DIECA 1 : 1) would all be different from each other and from the water control and this somewhat invalidates the comparison of activities.

Figure 3 illustrates the effects of IAA and DIECA separately and in admixture on the growth of wheat coleoptiles at a controlled-pH of 7. On the whole, DIECA is inhibitory except at the lowest concentrations. The mixture of 20 mg./l. IAA : 20 mg./l. DIECA is more stimulatory than IAA alone at this concentration but the large stimulation by IAA at 200 mg./l. is completely negated by 200 mg./l. DIECA. Thus both synergism and antagonism are displayed, depending on concentration. Since the results of experiments with mixtures of IAA and DIECA were often irregular and not highly reproducible, no further studies of this kind were made with the other chelating agents.

2. EDTA

The growth-promoting activity of EDTA was tested on wheat coleoptile segments over a concentration range of 0.002 to 200 mg./l. and a pH range of 4 to 8. Growth stimulation was observed at the lower pH values 4, 5 and 6. The concentration at which this stimulation occurs seems to be roughly inversely proportional to the pH (Figures 4, 5 and 6). In separate experiments at pH 4.3, concentrations of 0.002 and 0.2 mg./l. were stimulatory. At pH 5.1, 20 mg./l. was stimulatory, and at pH 6.5 stimulation was observed in separate experiments with either 20 mg./l. or 200 mg./l. However, in one experiment no stimulation at all was shown at pH 6. No concentration was stimulatory at any of the higher pH values, 7.1 to 8.1. Following Simon and Beevers (1952), if the concentration which gives maximum elongation is regarded as that required to produce a given response at each pH, then from Figure 7 it will be seen that the activity of EDTA falls off with increasing alkalinity. The importance of this fact will be dealt with in the discussion of these experiments.

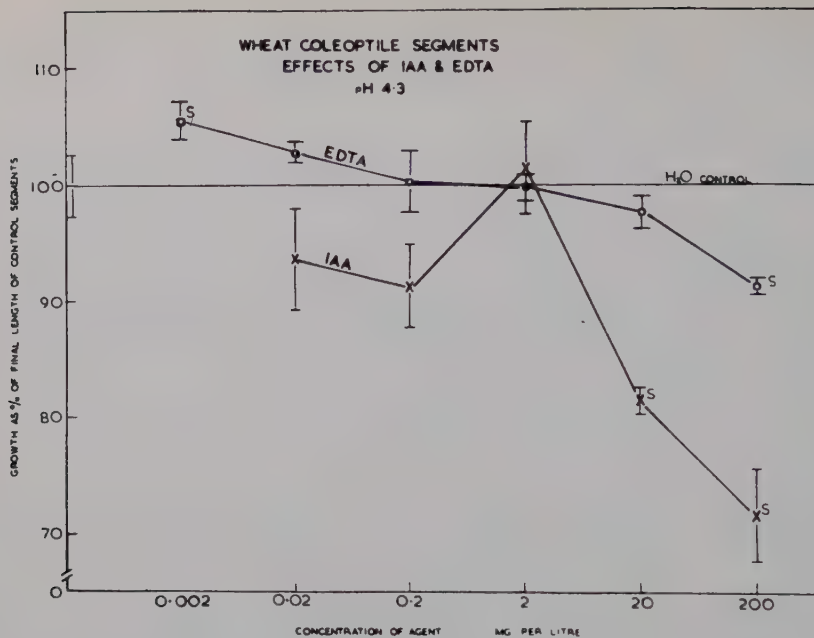


Figure 4.

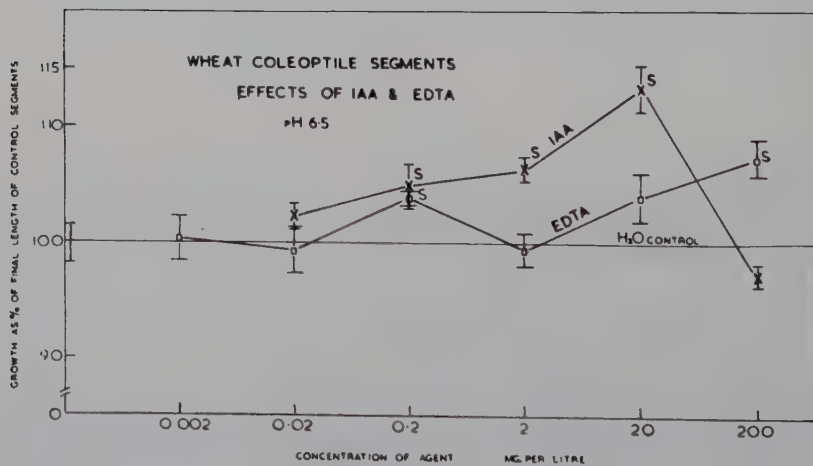


Figure 5.

Figures 4, 5 and 6. Effects of different concentrations (mgm./l.) EDTA and IAA on extension growth of wheat coleoptile segments. Figure 4, pH 4.3. Figure 5, pH 6.5. Figure 6, pH 7.3. For explanation of symbols see legend to Figures 1, 2 and 3.

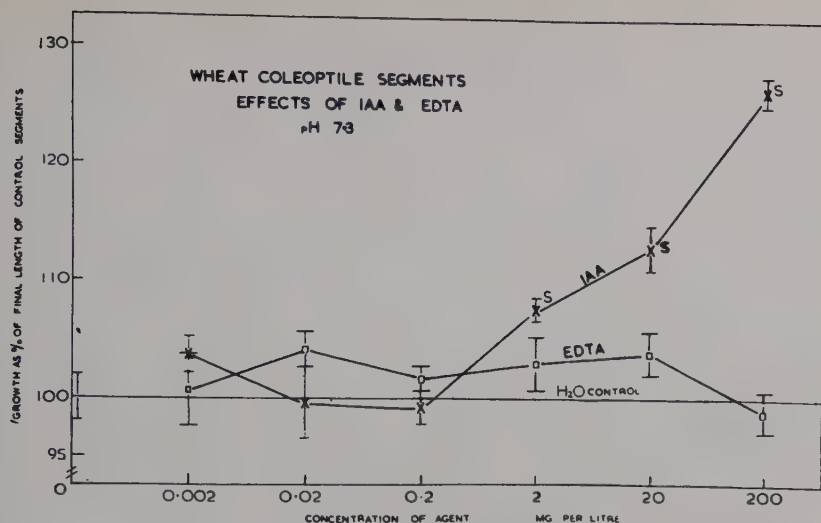


Figure 6.

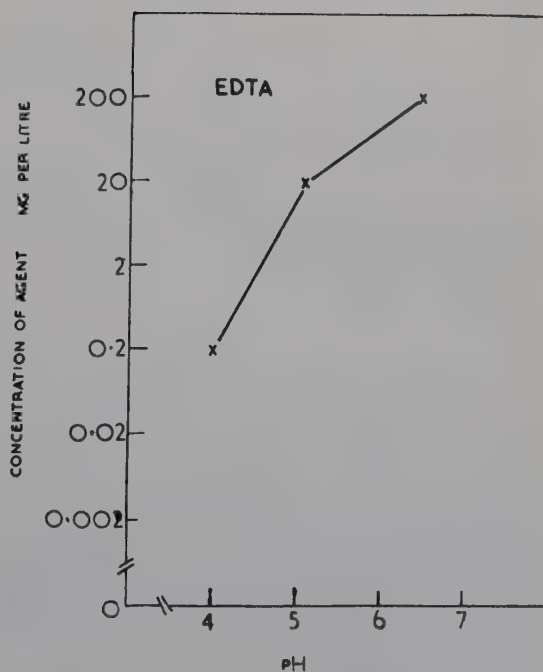


Figure 7. Concentrations of EDTA (mg./l.) required for maximal growth response of wheat coleoptile segments at different pH values.

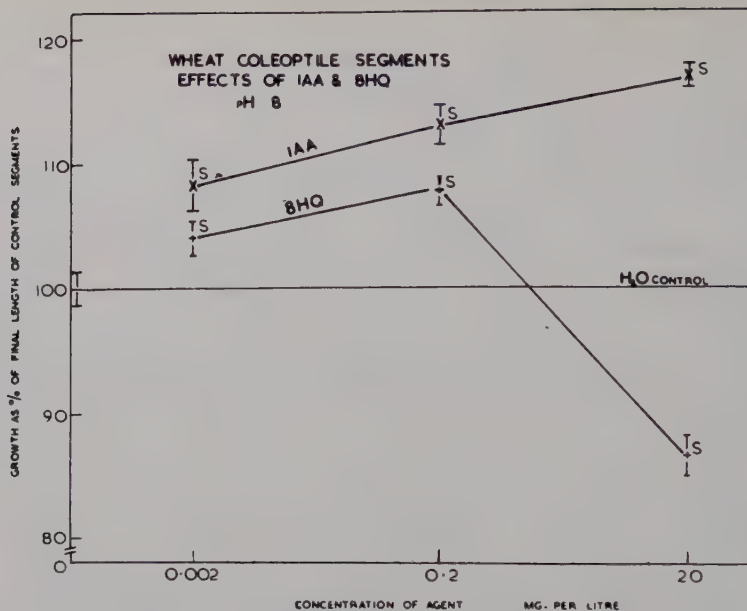


Figure 8. Effects of different concentrations of 8-HQ and IAA at pH 8 on extension growth of wheat coleoptile segments. For explanation of symbols see legend to Figures 1, 2 and 3.

3. 8-Hydroxyquinoline

In five experiments 8-HQ at a concentration of 20 mg./l. was inhibitory to the growth of wheat coleoptiles in the pH range 5.1 to 8.0. Lower concentrations (0.2 mg./l. and 0.002 mg./l. at pH 8; 0.002 mg./l. at pH 7 and 0.02 mg./l. at pH 4.1) were often stimulatory (Figure 8). No significant activity was found in two experiments at pH 5.1 and 6.2. but this seems to have been exceptional.

4. DTC

At the lower ranges of pH, 3.3 and 4.2, no activity of this compound on the extension growth of wheat coleoptiles could be detected at any concentration between 0.002 and 20 mg./l. At pH 5.2 only the highest of these concentrations was stimulatory, whereas at pH 6.3 and 7.2 (Figure 9) growth was promoted by 2 mg./l. and by 20 mg./l. DTC. Finally at pH 8.1 all concentrations between 0.002 and 20 mg./l. were stimulatory. In all cases where stimulation of growth occurred the optimal concentration was 20 mg./l., irrespective of the pH.

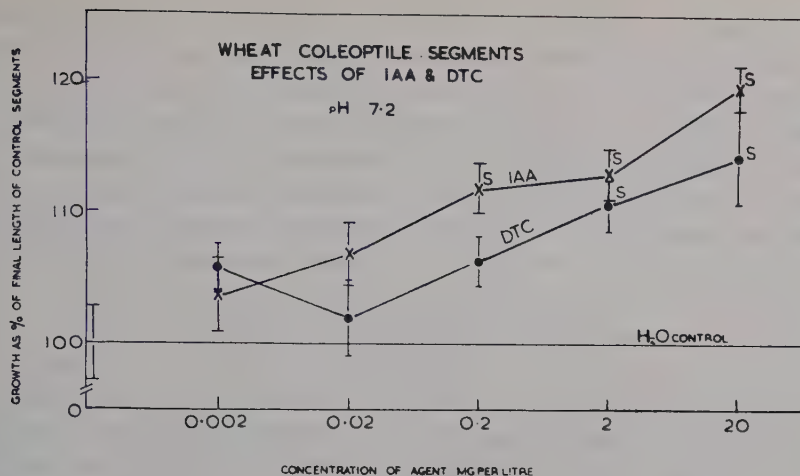


Figure 9. Effects of different concentrations of DTC and IAA at pH 7.2 on extension growth of wheat coleoptile segments.

Discussion

The decomposition of DIECA in acid solutions probably accounts for its inactivity or inhibitory activity in solutions buffered to pH 5 and 7. The slight activity in unbuffered test solutions and in solutions buffered to pH 8 is quite consistent with that obtained by Heath and Clark (1956 a) and by Fawcett *et al.* (1956) who used unbuffered solutions. The pH of an unbuffered 200 mg./l. solution of DIECA is about 8, a value which is somewhat unphysiological for the growth of coleoptiles. Albert and Gledhill (1947) have shown that DIECA reacts with a number of heavy metals (*e.g.* Mn, Co and Cu) at pH 7 and 37°C, but that there is no reaction with either calcium or magnesium under these conditions. It is therefore very unlikely that the rather small stimulatory effects of unbuffered DIECA are attributable to the sequestration of calcium from the cell wall. The effects of mixtures of IAA and DIECA are rather difficult to interpret and rather erratic, but the data given above do not support Heath and Clark's hypothesis because with different concentrations both synergism and antagonism are shown.

Unbuffered solutions of disodium-EDTA have a pH of about 4. It has been shown above that the activity of EDTA in promoting the growth of coleoptiles is greatest in the pH range 4—6. Weinstein *et al.* (1956 a) found that EDTA was stimulatory in unbuffered solutions and also in solutions buffered to pH 5 with phosphate buffer. Other authors (Bennet-Clark 1956, Heath and Clark 1956 a, and Fawcett *et al.* 1956) also found that unbuffered solutions

of EDTA stimulate coleoptile growth. The order of activity of EDTA in the experiments described above was much lower than that of IAA, and conforms rather with that found by Bennet-Clark and by Fawcett *et al.*, than with that found by Heath and Clark (1956 a) and by Weinstein *et al.* (1956).

The growth-promoting activity of EDTA declines with increasing alkalinity (Figure 7). We shall now show that this quite contrary to what may be expected on the basis of the calcium-chelation hypothesis. From the Law of Mass Action the stability constant (or affinity constant) K^a of a chelating reaction is

$$K^a = \frac{[\text{Metal chelate}]}{[\text{Metal ion}] [\text{Chelating agent}]}$$

Log K^a will be the value of pH at which half the total amount of the chelating agent will form the metal chelate. From the affinity constant of the calcium chelate (CaEDTA) one may calculate its degree of dissociation at a given pH. Log K^a CaEDTA at 25°C = 10.77 (Martell and Calvin 1952). For simplicity let us assume an approximate value of 11. Then, at pH 4,

$$\log \frac{[\text{Ca EDTA}]}{[\text{EDTA}]} = 4 - 11 = -7$$

The proportion of chelate to uncomplexed EDTA will be as 1 is to 10^7 , *i.e.* very little calcium will be chelated. At pH 6, similarly, the proportion will be as 1 to 10^5 . Theoretically, at pH 11 50 % of the EDTA will have complexed with calcium. Actually, the "water softening" activity of tetrasodium-EDTA reaches a maximum at about pH 9 (Martell and Calvin 1952). Thus in the pH range 4—6, where the growth-promoting activity of EDTA is at its highest, its calcium chelating activity is extremely low. Indeed, a pronounced calcium chelating activity would be obtainable only at a pH which would itself be somewhat toxic. These conclusions are, of course, in full agreement with the lack of ability of EDTA to sequester calcium from the cell wall at pH 5, which was demonstrated in the first paper of this series.

The activity of 8-HQ on the other hand, increases with increasing alkalinity, although its pK is 4.7. However it has been shown (Albert and McGrath 1947) that this compound does not react with calcium even at a pH of up to 8.6 at 37°C. Nevertheless, 8-HQ promotes the growth of coleoptiles at pH 7 and 8, possibly by virtue of its chelating properties, for it chelates a number of metals of biological importance within the pH range from 4 to 8 (Albert and McGrath 1947, Albert 1951).

The compound DTC is much more active than the chelating agents discussed above. In fact, its activity is of the same order as that of IAA. However, it is effective only in the pH range above 6.3 and its activity appears to increase with alkalinity. The results of Gordon and Moss (1958) who have

claimed that the auxin-like effects of DTC on oat coleoptile segments are insensitive to pH are explicable because they tested only unbuffered or buffered solutions in the pH range 3.6—5.0. Fawcett *et al.* (1956) believe that DTC induces a true auxin response in wheat coleoptiles and from the work of van der Kerk *et al.* (1955) (who discovered its auxin-activity), Gordon and Moss (1956) and Veldstra (1956) it is clear that DTC may be characterised as an auxin. However, Wain (private communication) believes that DTC has little or no activity as a chelating agent and that its growth-promoting activity cannot be due to chelation. On the other hand, it has recently been shown (Cohen, Ginzburg and Heitner-Wirguin 1958) that IAA does form chelates with copper and ferric ions. The stability constant of the copper chelate of IAA (5.5) is such as to render it possible that the action of IAA on plants might involve copper chelation. According to these authors, IAA does not form chelates with either calcium or magnesium. In view of this work, it would be interesting to see whether or not DTC has chelating properties similar to those of IAA.

Weinstein *et al.* (1956 a) believe that because the uncomplexed EDTA stimulates growth but not the Fe-EDTA chelate, support is lent to the calcium chelation hypothesis. This is not true. Their data do not constitute evidence in favour of calcium-chelation, but merely show that it is the uncomplexed chelating agent which is active. Although Heath and Clark (1956 a) did suggest that the chelating agents may sequester calcium from the cell wall, they thought that the probability that heavy metals involved in cell metabolism might be chelated was greater. The results we have obtained indicate that the hypothesis that it is calcium which is chelated, and that the chelating agents promote growth by sequestering calcium from the cell walls is not satisfactory. Nevertheless, it seems quite likely that the growth-promoting activity of the chelating agents does depend on their ability to form chelates. They may activate or inhibit enzymes by removing certain metals. More specifically they may act as inhibitors of the IAA-oxidase system since this system is known to contain either Cu or Fe in its prosthetic groups. In this connection it is interesting to recall the speculation of Chodat referred to the previous paper in this series, in which copper was mentioned. Terpstra (1953) claims that the enzymatic destruction of IAA during extraction from plant materials with water may be prevented by the presence of 0.01 % DIECA, which strongly chelates copper. Effects of chelating agents on enzymes involved in plant respiration have been reviewed by James (1953) and by Weinstein *et al.* (1956 b). Clearly the number of possible enzyme systems which might be affected is very large, and the amount of work which has been published is too small as yet to permit the formulation of any but working hypotheses.

Summary

Data are given from experiments on the effects of the chelating agents DIECA, EDTA and 8-HQ on extension growth of segments of oat and wheat coleoptiles at different pH values. DIECA and 8-HQ are inactive or slightly inhibitory at pH values below pH 7 but above this pH concentrations less than 2-mg./l. are somewhat stimulatory. The effects of mixtures (1:1) of DIECA and IAA on the growth of coleoptiles are shown to be complex, exhibiting both synergism and antagonism. The effects of DIECA and 8-HQ on growth are shown to be inexplicable in terms of the chelation of calcium.

EDTA does not promote growth at pH values above 6.5 and the activity of EDTA falls off with increasing alkalinity. This is contrary to what one would expect on the hypothesis that the growth promoting action of EDTA is due to the sequestration of calcium from the cell wall.

Previous reports of the auxin-like activity of DTC are confirmed. This compound is more active in alkaline than in acid solutions. It is suggested that it might have chelating properties similar to those of IAA.

The authors would like to thank Mr. L. Hollow for constructing the coleoptile microtome, Professor G. W. Leeper for discussions on chelating agents and Professor R. L. Wain, Wye College, Kent for a gift of DTC. The junior author held a C.S.I.R.O. Research Scholarship during the time when this work was carried out.

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The Uptake of Ions by Isolated Potato Tuber Mitochondria

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The suggestion that mitochondria may function in ion uptake was first made by Mullins (1940). His investigation showed that the protoplasmic granules of *Nitella* tended to concentrate radioactive potassium and phosphorus. His technique was repeated by Wernstedt (1944) with radioactive lead which resulted in similar findings. Later, Arisz (1945) proposed that microscopic particles may transport ions through the cytoplasm.

Since mitochondria had been found to contain the cytochrome components of the respiratory system, Robertson (1951) raised the possibility that mitochondria or microsomes may be the carriers of ions. He pointed out that their high lipid content would facilitate the transport of the ions across lipid membranes. It was subsequently found by Long and Levitt (1952) that mitochondria and microsomes of potato tuber slices which had been exposed to radioactive calcium or phosphate contained much higher concentration of these ions than the tissue as a whole or any other tissue fraction. Robertson, Wilkins, Hope, and Nestel (1955) later found that isolated beet and carrot mitochondria could accumulate sodium, potassium and chloride ions.

It was pointed out by Lundegårdh (1954) that although mitochondria may have membranes, as has been shown by electron photomicrographs, they "are apparently not built for the development of a high osmotic pressure" and would, therefore, not accumulate free ions to any great extent. He recognized that mitochondria could acquire an adsorbed layer of ions and could transport them "from one part of the cell to another by means of protoplasmic streaming or a more independent mobility of the mitochondria

themselves." Florell (1957) has presented evidence that calcium increases the formation of mitochondria which in turn cause increased ion uptake.

In this investigation the authors have studied the effects of temperature, time, pH, enzyme inhibitors and stable ions on the uptake of radioactive ions by isolated potato tuber mitochondria.

Materials

A hundred pound sack of potatoes (*Solanum tuberosum* L., variety Burbank Russets), was obtained in the fall of the year and stored in the cold room at 3°C. This amount was sufficient for all the investigations carried on during the year. Potatoes rather than other materials were chosen for this investigation because they were easily obtainable, they were probably genetically similar and when their outermost tissues were removed by paring, the remaining tissue was relatively homogeneous. Since mitochondria of different cell types may behave differently, this was a desired feature. In addition, previous investigations in this laboratory had been undertaken with potatoes, and the techniques and information obtained by these investigations could be of benefit.

Radioactive calcium-45, rubidium-86 and phosphorus-32 were obtained from the Oak Ridge National Laboratory. Calcium and rubidium were used in the form of chlorides while the phosphorus was used as phosphoric acid. For some of the chemicals employed the following abbreviations are used: 2,4-dinitrophenol (DNP), trihydroxymethylaminomethane (TRIS), adenosinetriphosphate (ATP) and adenosinemonophosphate (AMP).

Methods

All of the isolations were performed at 3°C in a cold room. A random sample of potatoes was pared until 550 grams were obtained. One half of this amount was diced and placed in a Waring blender containing 125 ml. 0.5 *M* sucrose and sufficient buffer to produce a pH of 6.7—7.0 (as determined by a glass electrode) when the potatoes were blended. The mixture was ground with the blender for about 30 seconds and filtered simultaneously through two Buchner funnels (covered by No. 1 Whatman filter paper, 11.0 cm. diameter) under mild suction. The other half was treated in a similar manner. The resulting filtrates were poured into sixteen plastic centrifuge tubes which were then subjected to a force of 1—2,000×*G* for 20 minutes with an Ivan Sorvall SS2 vacuum centrifuge. A pellet, containing starch, and other cell material which had been able to pass through the filter paper, formed in each tube. The supernatant was then poured into sixteen other plastic tubes and centrifuged at 14,500×*G* for 10 minutes, which resulted in mitochondrial pellets. The supernatant was decanted and its pH determined before discarding. The pellets were homogenized in their tubes with rubber policemen. To each tube 1 ml of radioactive solution, which was buffered to about pH 7, was added, and the mitochondria and solution were mixed. The radioactive solution was less than 10⁻² *M* since preliminary experiments showed saturation of the mitochondria did not occur below this value. The tubes were placed on their sides (the outside lips of

the tubes prevented the solution from running out) to give a large solution surface for gas exchange. To prevent evaporation, they were then placed in a watervapor saturated environment under glass bell jars. The tubes were left for varying lengths of time at 3°C or room temperature (24°C). At the end of this time, 10 ml of cold 0.5 M sucrose (pH 7) were added to each of the tubes and the tubes were centrifuged at 14,500×G for 10 minutes to separate the mitochondria. The supernatant was decanted and the tubes, containing the mitochondrial pellets, were allowed to drain. The mitochondria were transferred with the aid of rubber policemen, into previously weighed 10 ml. beakers which in turn were placed in a vacuum desiccator containing activated alumina (Al₂O₃). The desiccator was then evacuated to about 0.5 mm Hg and left overnight. The next day the beakers were removed and the dry weights of the mitochondria were determined. The mitochondria were wet-washed over a hot plate and the radioactivities were determined.

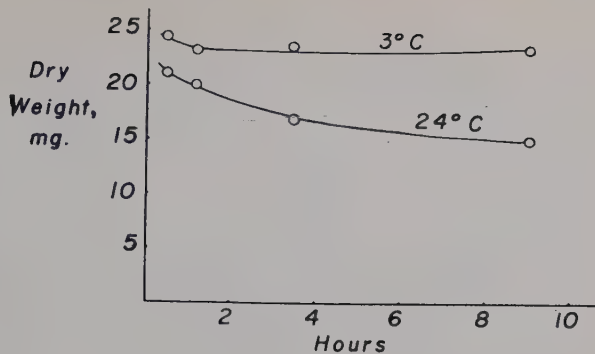
This procedure was varied by employing different buffers *i.e.*, potassium phosphate, arginine, or TRIS; by adding various inhibitors; by adding substrates, or stable ions; and by varying the pH.

An attempt was made to determine if a specific mitochondrial component could be shown to have a strong affinity for the radioactive ions. After the mitochondria had been exposed to the radioactivity, they were dried as mentioned above and extracted with ether-alcohol (1:1). The solvent containing the lipids was pipetted out, placed in planchets, dried and its radioactivity measured. To the lipidless residue was added enough one per cent sodium dodecylsulfate solution of pH 8.5 to give an approximately one per cent mitochondrial suspension. This was maintained at 40°C for about 20 hours. As a result of this treatment (Fraenkel-Conrat and Williams, 1955) the nucleo-proteins were broken down into their nucleic acid and protein components. Ammonium sulfate was added to give 35 per cent saturation and the precipitated protein was separated by centrifugation. The supernatant was placed at 3°C for a day and a half. This resulted in the crystallization of most of the nucleic acid fraction. The radioactivities of the lipid, protein, nucleic acid and supernatant portions were determined with a Geiger counter after they were digested by acid.

Results

It is known that mitochondria are very labile and do not retain their enzymatic capabilities for long periods of time (Laties 1953, Millerd 1953, Price and Thimann 1954, and Sharpsteen and Conn 1954). This may be due to enzyme inactivation or to loss of enzymes. Figure 1 shows the effects of time and temperature on the dry weights of potato mitochondria. There was a definite loss at both 3° and 24°, even for periods of less than an hour. This was probably due either to autolysis and solubilization of part of the mitochondria or to the breakdown of the mitochondrial membrane with a release of soluble proteins. This loss of dry weight did not appear to decrease the uptake of calcium until after 3 hours, as Figure 2 shows. With rubidium and phosphate (Figure 3) there seemed to be a leveling off of the uptake

Figure 1. Dry weights of mitochondria isolated in 0.5 M sucrose plus 0.1 M potassium phosphate buffer and left for varying lengths of time before final centrifugation.



after the first 15 minutes or so. The decrease in activity after a certain time at 24°C was presumably due to mitochondrial damage.

Figure 2 shows a typical curve obtained when a TRIS-sucrose mitochondrial isolation medium is used and the radioactive calcium uptake vs. time at 24°C is plotted. The curve illustrates the first rapid uptake followed by a more gradual accumulation which in time changes to a loss. These three phases may be due to adsorption, active uptake and mitochondrial damage. The time of maximum uptake was sometimes less and sometimes more than the three hours shown in Figure 2. The uptake seems to depend on the condition of the potatoes and the isolation medium. Very little of this radioactive calcium is exchangeable with stable calcium. The ion uptake by mitochondria isolated at varying acidities is shown in Table 1. Each fraction was presumed to contain the same amount of mitochondria initially since they were isolated from equal quantities of potatoes. More material, however, was centrifuged down from solutions of low pH values than from solutions of higher pH values. Judging from earlier investigations, this was due to precipitation of soluble proteins at the low pH. The counts per minute of the calcium taken up did not vary tremendously with the different acidities.

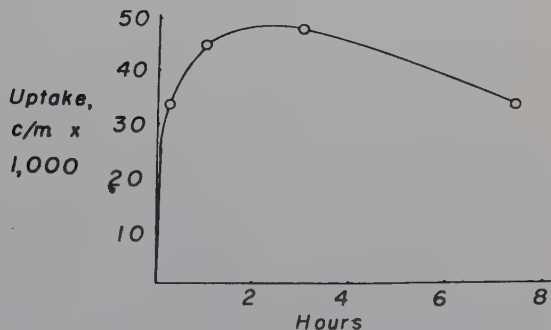


Figure 2. Uptake of radioactive calcium by mitochondria from 7.5×10^{-3} M CaCl_2 at 24°C. Isolation as in Fig. 1.

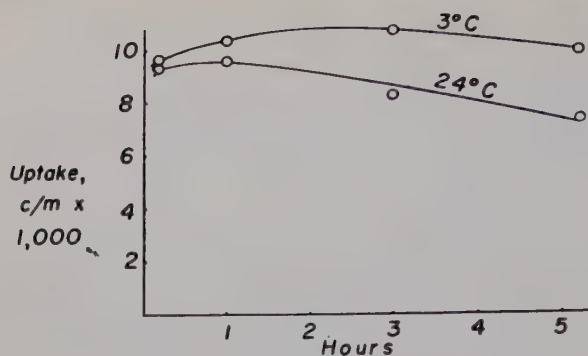


Figure 3. Uptake of radioactive phosphorus by mitochondria isolated in TRIS-sucrose and exposed to 10^{-9} M inorganic P^{32} for varying lengths of time.

This seems to indicate either (1) the material which combines with calcium was present in almost equal quantities at the various acidities; or (2) the binding material may not be present in equal amounts, but the lack of material at high pH values was compensated for by the development of larger calcium-attracting charge on the substances that *were* present. A slightly different trend of uptake was observed with rubidium. A neutral pH appeared to be most conducive to rubidium uptake. The reason may lie in better maintenance of the integrity of the rubidium-combining material at a neutral pH.

It has been found that much more radioactive calcium is taken up by mitochondria at room temperature (24°C) than in the cold (3°C), (Shean, 1956). Further work has shown that whereas the uptake at 3°C leveled off after the first few minutes, the uptake at 24°C continued until the mitochondria became damaged. The uptake of calcium at 24°C was found at times to be over five times the uptake at 3°C . The ratio of the two depended on the duration of the time the mitochondria were in the radioactive solution, the time since the potatoes had been harvested and the concentration of the radioactive solution. These findings may indicate that metabolic

Table 1. Calcium and rubidium uptake by mitochondria isolated at various acidities.

Isolation medium pH	Calcium			Rubidium		
	Pellet dry weight-mg	c/m	c/m · mg	Pellet dry weight-mg	c/m	c/m · mg
5.85	107	10,985	102	113	26,835	238
6.8	64	8,710	136	53	29,990	565
7.65	31	9,180	296	34	18,590	547

0.5 M sucrose and TRIS used in isolation medium. Exposed to 10^{-4} M Rb^*Cl for three hours at 3°C .

Table 2. *Rubidium and calcium uptake at 3°C and 24°C.*

Conditions	Rubidium		Calcium	
	c/m	c/m · mg of dry wt.	c/m	c/m · mg of dry wt.
Mitochondria at 3°C	29,200	667	7,800	175
Mitochondria at 24°C	19,400	660	20,100	558

Mitochondria were isolated in TRIS-sucrose and were exposed for two hours and fifty minutes in 1 ml. 10^{-4} M Rb^+Cl or 1 ml. 10^{-4} M Ca^+Cl_2 . Some then were placed in 10 ml. 5×10^{-3} M stable CaCl_2 , 0.5 M sucrose and centrifuged. Others were centrifuged only in 10 ml., 0.5 M sucrose.

activity or a chemical reaction may be responsible for the calcium uptake. They also may indicate that protein is binding the calcium since Austin, Sunderman and Camack (1927) showed that the cation binding power of protein decreases with a decrease in temperature. But this explanation seems less likely since another cation (Rb) failed to give the same results.

Experiments were performed to determine how rubidium uptake responded to 3° and 24° temperatures. Table 2 shows that unlike calcium uptake, rubidium uptake is greater at 3°C than at 24°C. This may be explained by (1) a metabolic uptake or chemical bonding of calcium and an adsorptive uptake of rubidium, (2) the breaking down of rubidium combining sites at warm temperatures and a building up of calcium combining sites, (3) the mitochondrial dry weight that was lost at 24°C consisted of material that bound rubidium but did not bind calcium. (However, the constancy of the uptake of rubidium per dry weight at the two temperatures does not seem to indicate this for rubidium.), (4) the rubidium bound at 24°C was released during the final centrifuging at 3°C into the supernatant, *i.e.*, energy is required to maintain the rubidium taken up. In some experiments calcium was accumulated in the mitochondria to 11.5 times the concentration of the external medium at 24°C while rubidium was not of a higher concentration in the mitochondria than in the medium. Phosphate uptake appeared to be similar to rubidium uptake in that it was somewhat greater at the lower than at the higher temperature.

Neither dinitrophenol nor sodium fluoride at concentrations of 10^{-3} M at about pH 7 caused a significant change in rubidium uptake by mitochondria at 3°C or 24°C. Dinitrophenol did not greatly affect calcium uptake either at 3°C or 24°C. At 3°C the uptake was inhibited a questionable 1 % while at 24°C it was inhibited about 7 %. Although the need for high energy phosphates for salt accumulation by tissues has been demonstrated by Robertson, Wilkins and Weeks (1951), these results with dinitrophenol do not rule out the possibility that mitochondria may be involved in active ion uptake by

Table 3. *The effect of washing potato slices before extracting the mitochondria on the calcium uptake by the isolated mitochondria. Mitochondria placed in Ca^{45} for 1 hour. Sucrose and phosphate buffer used for isolation.*

Mitochondria from	Uptake of Calcium c/m · mg
Slices washed 19 hours at 18°C	
Uptake at 3°C	31
Uptake at 24°C	67
Slices not washed	
Uptake at 3°C	22
Uptake at 24°C	49

plants. They do seem to indicate that mitochondria do not require high energy phosphates in order to take up ions themselves.

Sutcliffe (1952) has demonstrated that tissue washed in running water for a number of hours is able to accumulate ions at a greater rate than unwashed tissue. Laties (1954) has observed, however, that the mitochondria of tissue washed at room temperature may be adversely affected so that some enzymes are inactivated, especially if the mitochondria are isolated in hypotonic solutions. He has suggested that washing may act similarly to DNP in uncoupling phosphorylation from respiration. This, however, would not agree with the results obtained by tissue washing since DNP decreases the uptake of ions by tissue as Robertson et al. (1951) have demonstrated. Potato slices washed in running water at 18°C for 19 hours and potatoes not washed but left at 3°C indicated that the mitochondria of the washed potatoes accumulated calcium at a slightly greater rate than the mitochondria of the unwashed potatoes whether the uptake was at 3°C or 24°C (Table 3). Whether this was due to the washing or the increased temperature during the washing is uncertain. The increased uptake by mitochondria of washed tissues agrees with the findings that increased salt uptake occurs with washed tissue. Perhaps, as Skelding and Rees (1952) have pointed out, an inhibitor of ion absorption had been washed away which enabled more calcium to be taken up by the mitochondria of washed tissue than by the mitochondria of unwashed tissue. Or perhaps the cutting of the tissue permitted new ion-binding compounds to form inside the cells.

In an effort to determine whether the combining sites for rubidium were dependent solely upon electrostatic forces or whether they were specific for groups of physiological isotopes, experiments were performed in which the previously accumulated rubidium was given an opportunity to exchange during the final centrifugation with its physiological isotope potassium, or with stable calcium. The results, as shown in Table 4, indicate that both stable calcium and potassium cause an increase in the rubidium uptake at

Table 4. *The uptake of rubidium by mitochondria in the presence and absence of calcium and potassium.*

Rubidium uptake at	c/m	Dry weight	c/m · mg
3°C	838	52.0	16
24°C	327	30.0	11
3°C plus 2×10^{-2} M stable CaCl_2 ...	1,179	52.4	23
24°C plus 2×10^{-2} M stable CaCl_2 ...	740	43.2	17
3°C plus 2×10^{-2} M stable KCl	1,175	50.9	23
24°C plus 2×10^{-2} M stable KCl	688	34.4	20

Mitochondria exposed to 10^{-4} M Rb^*Cl for 70 minutes, then centrifuged in 0.5 M sucrose with and without the stable Ca^{++} or K^+ . Done in September with the previous year's potatoes.

both 3°C and 24°C. This was certainly not expected. It might have been expected that one or both of the stable cations would exchange with the radioactive rubidium and that a decrease in rubidium uptake would therefore take place. That such did not occur is further evidence that the absorption by the isolated mitochondria may be closely related to active absorption and is not a simple adsorption process. Calcium may have caused a tightening of the Rb-carrier bond as Kahn and Hanson (1957) have suggested so that the rubidium ions were not washed off as easily during the final centrifugation. The physiological state of the potatoes is probably very important in uptake studies. In these experiments the potatoes had been kept at 3°C for almost a year. They were still turgid and outwardly appeared normal. The effect of calcium on rubidium uptake was not as pronounced if the potatoes used were recently harvested.

It has been shown by Sharpsteen and Conn (1954) that the presence of cysteine in the isolation medium helps preserve the enzymatic capabilities of potato mitochondria. They also found that maximum enzymatic activity occurred if a reaction solution of ATP, AMP, magnesium ions, phosphate ions, and succinic acid were present. The use of cysteine and the reaction solution (Table 5) did not, however, enhance the uptake of rubidium or calcium by potato mitochondria. These results seem to indicate that the enzymes which are protected and promoted by the cysteine and the reaction solution are not involved in the ion uptake of calcium or rubidium by potato mitochondria.

From the fractionation of the mitochondria no conclusive evidence was obtained to pinpoint a certain fraction as the one combining with the calcium. Different experiments gave different results. The reason for this inability to pinpoint ion-combining compounds could be due to breakdown of the combining compound (*e.g.*, nucleo-protein) during fractionation, or to break-

Table 5. *Mitochondrial uptake of calcium and rubidium in the presence and absence of cysteine and a reaction solution containing cofactors.*

Treatment	Ca* uptake c/m · mg	Rb* uptake c/m · mg
Cysteine used in isolation medium		
with reaction solution	76	16.4
without reaction solution	87	17.8
No cysteine used in isolation medium		
with reaction solution	93	17.4
without reaction solution	91	18.3

For some an isolation medium of 0.01 *M* cysteine was used with the TRIS-0.5 *M* sucrose. The mitochondria were exposed twenty minutes at 24°C in 7×10^{-5} *M* Rb* or 7×10^{-5} *M* Ca*. The reaction solution consisted of 2×10^{-4} *M* ADP, 2×10^{-4} *M* AMP, 2×10^{-4} *M* MgH PO₄, 4×10^{-3} *M* succinic acid and 0.5 *M* sucrose buffered with TRIS to pH 6.8.

down of the membrane (if the ions were inside), during the treatment. There is a possibility that the ion-combining material will never be isolated by this method since it may induce ion release analogous to the postulated means of releasing ions into the vacuole.

In all of these experiments it was noted that certain uptake measurements changed according to whether the potatoes used were recently harvested or had been stored for a long time.

The isolation method cannot, of course, be expected to yield absolutely normal mitochondria. Some investigators, for instance, added versene to help maintain them in the more nearly normal state. But since Ca was the main ion investigated, versene could not be used in these experiments.

Discussion

The question arises as to whether the uptake of ions by free mitochondria is in any way related to active absorption by tissues as a whole.

The two resemble each other in the following respects:

- 1) Similarities in absorption curves for tissues and for free mitochondria (until mitochondrial damage occurs).
- 2) Greater uptake of calcium by tissues and free mitochondria at 24°C than at 3°C.
- 3) Saturation of tissues and free mitochondria occurs at about the same concentration of external medium (10^{-2} *M* CaCl₂).
- 4) Calcium appears to enhance rubidium uptake rather than to replace rubidium as would occur in simple adsorption.
- 5) Prewashing enhances uptake by tissue slices and free mitochondria.

The following differences between active uptake by tissues and uptake by free mitochondria were found:

1) Lack of inhibition of ion uptake by free mitochondria when 2,4-DNP or NaF is applied. This could, however, merely indicate that the enzymes inhibited by these compounds are not necessary for the part of ion absorption in which mitochondria function.

2) There seems to be no competition between rubidium and potassium.

3) Rubidium and phosphate uptake by free mitochondria are not increased by higher temperatures. This may mean that the calcium binding sites are more stable and therefore are retained in free mitochondria. Rubidium and phosphate sites may be more labile and, therefore, are destroyed during the process of isolation.

These results do not prove that mitochondria are associated with active ion uptake, but they provide sufficient evidence to encourage further investigations.

Summary

1. The isolated mitochondria show a progressive loss in dry weight that is more pronounced at 24°C than at 3°C.

2. This loss in dry weight fails to prevent a marked uptake of Ca^{45} during the first three hours but may explain the subsequent drop in Ca^{45} content.

3. The calcium uptake was markedly greater at 24°C than at 3°C; rubidium and phosphate uptake slightly greater at 3°C.

4. Dinitrophenol and sodium fluoride did not significantly affect ion uptake.

5. There was little exchange of radioactive rubidium for potassium or calcium.

6. The use of cysteine in the isolation medium or ATP, AMP, magnesium ions, phosphate ions and succinic acid in the radioactive solution did not increase the uptake of rubidium or calcium.

7. No calcium combining substance could be isolated by fractionating mitochondria which had taken up calcium.

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Drought Tolerance and Avoidance in Two Species of Oak

By

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Quercus palustris is normally found growing in the wetter areas, along streams or drainage ditches, near ponds or near swampy areas, while *Quercus rubra* grows predominantly on well drained uplands. One possible explanation of this difference in habitat is a difference in drought resistance — either tolerance or avoidance. The purpose of this investigation was to determine whether any such difference occurs when the trees are subjected to periods of water deficiency.

Materials and Methods

A. *Quercus palustris*

In early April, 1957, forty *Quercus palustris* seedlings, approximately eighteen inches in height and four to five years old, were collected in eastern Missouri, brought into the greenhouse, potted into six inch pots and watered enough to keep the soil in the pots moist. Starting on June 10, twenty of the seedlings were subjected to drought, twenty were watered as often as necessary to keep the soil near field capacity at all times. The latter normally required a daily filling of the pots to capacity (approximately one to one and one-fourth inches of water per pot). Occasionally, when the relative humidity of the atmosphere was high, a day or two would lapse without watering; however, during most of the experiment, the undroughted were watered at least once daily. In this way, they were never permitted to wilt. The droughted group was not watered until the leaves of the plants showed signs of permanent wilting — on an average every nine days — at which time the pots were watered to field capacity.

By August 1, ten of the droughted trees were severely damaged by the droughting treatment. Their leaves turned brown or yellow, curled and, in some cases, dropped

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from the plants. The margins and tips of the leaves were the first to be affected. The other ten droughted trees remained green and healthy except for an occasional browning of the leaf tips and margins. The effect on the tips and margins was observed to occur equally on the older mature leaves as well as on the new leaves that were formed after being brought into the greenhouse. Only those ten plants that had healthy green leaves were used in the subsequent experiments.

At the start of the experiment, most of the young trees were forming new leaves, approximately four per branch. In the droughted groups these new leaves continued to grow and develop during the first two or three weeks of droughting; but the plants failed to develop any new leaves subsequent to this period, while during the same period, the undroughted plants developed an average of eight new leaves per branch.

As fall approached, the droughted plants dropped their leaves or they turned brown earlier than the undroughted ones. The majority of the droughted trees had dropped their leaves by October 1 with the exception of three or four trees, whereas the undroughted trees did not drop their leaves nor turn brown until the latter part of October or the early part of November.

B. *Quercus rubra*

These plants were collected at the same time and in the same general area of the State as the seedlings of *Q. palustris* and potted at the same time. Like the latter, they were watered normally until the time of droughting. Eight plants were droughted and eight were kept profusely watered as previously described. This species did not respond to the abundant water supply as did the first mentioned species; that is, they did not show the same excessive growth over the droughted group as in the case of *Q. palustris*.

Essentially the same response was observed in the droughted group as in the case of *Q. palustris*. The drought injury was first noticed at the tips of the leaves and along the margins. There was no significant ability of this species to survive longer desiccation periods than *Q. palustris*. The plants required watering at approximately the same dates as those of the latter species. They were also held as near to the permanent wilting point as possible, and, again, one-half of the trees did not survive the droughting treatment. The others remained green and apparently normal except for the lack of abundant growth, although there was more leaf enlargement than in the case of *Q. palustris*.

It must be mentioned here that these observations of drought survival apply only to the leaves and do not necessarily imply the ability of the roots and stems of the two different species to survive the same amount of water loss. This experiment was primarily directed toward determining the ability of the leaves of the species to harden to drought.

C. Investigation of drought tolerance

After half of the seedlings were droughted for a few weeks, the following procedure was used to determine whether the droughted plants had become hardened to drought.

In the first series the sixth leaf, counting from the bottom upward, was removed between 8.00 and 10.00 a.m. and taken immediately to the laboratory where it was

sectioned. In later experiments the third and ninth leaves were used. The laminae were supported between pith sticks and transverse sections made by hand. These were then placed in chambers having controlled relative humidities ranging from 100 to 90 per cent. A total of eight to ten sections was made for each relative humidity chamber.

Difficulty was encountered in the first three or four attempts to keep the sections alive even in the control chamber of 100 per cent relative humidity. It was soon discovered that when the thin sections were floated off the razor blade into water and then lifted onto the chamber lid, every cell survived for many hours in the control chamber; whereas, if the sections were placed directly from the blade onto the lid they failed to survive. Apparently a low moisture content that was fatal to the cells was reached before they came to equilibrium with the atmosphere and solution in the chamber and the additional moistening aided in keeping the cells alive until equilibrium was reached. This method was followed throughout the experiment. Later, some difficulty was again encountered in keeping the control sections (in 100 per cent relative humidity) alive. This was corrected when a new razor blade was used with each series of sectioning.

The relative humidity chambers used were small preparation dishes with lids. Each was about one-fourth filled with a known sucrose solution. Graded concentrations were used to obtain the desired relative humidities in the atmospheres above them according to Walter (1924) and Weismann (1939) (see Levitt 1956). The sections were placed on the inside of the lid of the preparation dish and the lid sealed with vaseline.

After the sections were placed in the chambers in the morning and the lid sealed on, they were left for twenty-four hours in a dark room having a relatively constant temperature ($\pm 1-2^{\circ}\text{C}$). This allowed the exposed cells to approach equilibrium with the atmosphere of the relative humidity chambers. The following morning the sections were removed from the chambers by washing them off the lids with an eye dropper into a solution of 12 parts per million neutral red in 0.1 molar CaCl_2 . This was covered and left standing for thirty minutes. At the end of this period, they were observed microscopically for vital staining of the mesophyll cells. The palisade layer was particularly suitable for observation since it always stained uniformly.

D. Investigation of drought avoidance

The droughted and undroughted plants were next investigated to determine whether the droughted plants developed a mechanism to avoid loss of moisture from their leaves to the surrounding atmosphere. The leaves were obtained from the same plants that were tested for drought tolerance. Most of the trees now had the sixth leaf removed, some the third and ninth, from the earlier experiments on tolerance. The two trees to be worked with were taken into the laboratory from the greenhouse the night before the experiment was started and each tree was watered at this time. The following morning one leaf (usually the eleventh or twelfth) was removed from each tree and immediately weighed. They were then suspended in air by means of a fine string attached to the petiole and to the beam of a balance. Two sets of precision laboratory balances were used for this experiment. They were placed side by side away from any windows and doors of the laboratory in order to avoid drafts, and the windows and doors were kept closed as much as possible during these

experiments. The light was kept as uniform as possible on the two leaves. To avoid any possible difference due to the positions of the two balances, the undroughted and droughted were alternated with each successive experiment. There was some variation in temperature and relative humidity, but this must have affected each leaf equally. An outline of each leaf was traced on paper and the area determined with a planimeter. The weights of the two leaves were then taken periodically until equilibrium was reached with the room atmosphere.

Results

A. Drought tolerance of Q. palustris

The droughted sections frequently survived in a relative humidity of 94 per cent, and in three instances one quarter to one half of the cells survived as low as 92 per cent (Table 1). Only on two occasions did any of the undroughted survive as low as 94 per cent, and even then this was true of only a few of the cells. Frequently only half or less of the undroughted cells survived equilibrium with a relative humidity of 96 per cent. None of the cells from either droughted or undroughted plants survived in the 90 per cent chamber. There was a slight increase in the drought tolerance of the droughted plants as the drought progressed. In the early part of the experiment only part of the cells survived a relative humidity of 94 per cent. By comparison, on July 22, $\frac{1}{4}$ to $\frac{1}{2}$ of the cells survived in a relative humidity of 92 per cent.

It was also interesting to note that on several occasions every cell of every section of the undroughted was living at a relative humidity of 96 per cent,

Table 1. *Survival of leaf sections of droughted and undroughted Quercus palustris seedlings after 24 hours in the graded relative humidity chambers.*

Date	Treatment	Per cent relative humidity					
		100	98	96	94	92	90
6.18.57	Droughted	all	all	all	$\frac{1}{2}$	0	0
	Undroughted	0	0	0	0	0	0 ¹
7. 4.57	Droughted	all	all	$\frac{3}{4}$	$\frac{1}{4}$	0	0
	Undroughted	all	all	$\frac{3}{4}$	0	0	0
7. 8.57	Droughted	all	all	$\frac{3}{4}$	$\frac{1}{4}$	0	0
	Undroughted	all	$\frac{3}{4}$	$\frac{1}{2}$	0	0	0
7.11.57	Droughted	all	all	all	all	$\frac{1}{4}$	0
	Undroughted	all	all	all	0	0	0
7.17.57	Droughted	all	all	$\frac{3}{4}$	$\frac{1}{2}$	0	0
	Undroughted	all	all	all	0	0	0
7.22.57	Droughted	all	all	all	all	$\frac{1}{4}$	0
	Undroughted	all	all	$\frac{3}{4}$	$\frac{1}{4}$	0	0
7.24.57	Droughted	all	all	all	$\frac{3}{4}$	$\frac{1}{2}$	0
	Undroughted	all	all	$\frac{1}{4}$	few cells	0	0

¹ Cells died when not dipped in water before placing in the relative humidity chambers.

Table 2. *Survival of leaf sections of droughted and undroughted Quercus rubra seedlings after 24 hours in the graded relative humidity chambers.*

Date	Treatment	Per cent relative humidity					
		100	98	96	94	92	90
8.14.57	Droughted	all	all	all	all	1/4	0
	Droughted	all	all	all	all	3/4	0
8.19.57	Undroughted	all	all	all	0	0	0
	Undroughted	all	all	all	0	0	0
8.22.57	Droughted	all	all	all	all	0	0
	Undroughted	all	all	all	0	0	0
8.27.57	Droughted	all	all	all	all	0	0
	Droughted	all	all	all	3/4	0	0
8.29.57	Droughted	all	all	all	all	1/2	0
	Undroughted	all	all	all	3/4	0	0
9. 4.57	Undroughted	all	all	all	all	1/2	0
	Undroughted	all	all	all	all	0	0

but every cell was dead at 94 per cent. Apparently there was a very sharp critical point in this range where moisture deficit was fatal. Tests with older and younger leaves than the sixth all agreed in yielding a greater drought tolerance of 2 % for the droughted leaves.

B. Drought tolerance of *Quercus rubra*

These seedlings had been droughted since June 21 in the same manner as those of *Q. palustris*, but the sectioning and exposure of the sections to the series of relative humidities was not begun until about the middle of August. Therefore, they were subjected to the drought for a longer period than the *Q. palustris* seedlings. If there is a marked difference in the ability of the two species to harden to drought, it should be evident in this study. The first five sets of results were similar to those obtained with *Q. palustris* (Table 2). Nearly all the cells of the droughted sections survived in 94 per cent relative humidity. Occasionally some cells would survive in the 92 per cent chamber. The undroughted all survived when in 96 per cent relative humidity, but they usually failed to live in a lower humidity. This was about the same two per cent difference that was found in the case of *Q. palustris*. The sixth set of undroughted leaves show greater drought tolerance, but these were obtained late in the summer after a spell of cool, dry weather.

C. Drought avoidance of *Quercus palustris*

There are several ways that a plant might maintain its moisture balance when subjected to conditions of reduced water supply. One of these is a

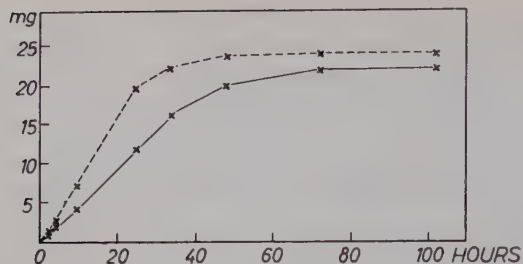


Figure 1.

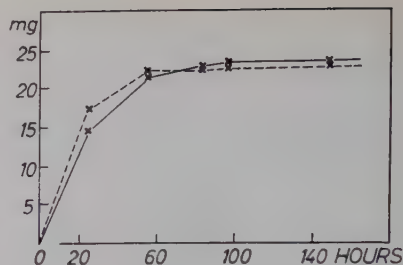


Figure 2.

Figure 1. Loss of water in mg. per sq. in. of leaf surface from undroughted (---) and droughted (—) excised *Q. palustris* leaves.

Figure 2. Loss of water from undroughted (---) and droughted (—) *Q. rubra* excised leaves.

reduction in cuticular transpiration from the leaves. The purpose of this experiment was to determine whether there was such a retardation of water loss from the droughted species under study.

The total water lost per square inch of leaf surface was slightly greater in the case of the undroughted leaves (Fig. 1), though this was mainly during the first few hours. In another experiment greater loss from the undroughted again occurred during the first twenty-four hours, but the loss soon leveled off and during the next twenty-four hours more was lost from the droughted, resulting in a slightly greater total loss from the droughted.

D. Drought Avoidance of *Quercus rubra*

A similar test was conducted using droughted and undroughted plants of *Quercus rubra*. The results were essentially the same as with *Q. palustris* (Fig. 2). There was a greater loss during the first twenty-four hours from the undroughted, equilibrium was nearly reached in forty-eight hours in both the droughted and undroughted, and the total loss was almost equal. Thus, there was essentially no difference in the abilities of the droughted plants of the two species to avoid loss of water through cuticular transpiration. The experiment was repeated several times.

Discussion

Some drought hardening occurred when the trees were exposed to a prolonged drought, but the hardening was the same in both species — *i.e.* an increased drought tolerance of 2 %. While this hardening could not explain

any difference in the ability of the two species to inhabit drier areas, it is conceivable that this 2 per cent difference would aid both in surviving a period of low available moisture, since it involved a relative increase in drought tolerance of 50 %.

A slight increase in drought avoidance also occurred due to droughting, since transpiration rate was slightly reduced. But again there was no difference between the two species. Furthermore, this decreased transpiration may have been more apparent than real, since the undroughted leaves contained more water (60.2 %) than the droughted (57.5 %).

Summary

1. Drought tolerance and avoidance of *Q. palustris* and *Q. rubra* were determined in an attempt to explain the more common occurrence of *Q. palustris* in moist areas, of *Q. rubra* in uplands.
2. In both species, the leaves of undroughted controls survived no lower than 96 % relative humidity: those that had been droughted survived 94 % and sometimes 92 % relative humidity.
3. In both species, the undroughted leaves showed a slightly greater water loss. This may have been due to the slightly lower water content of the droughted leaves.
4. There was no difference between the drought resistance of the leaves of the two species — whether they were compared for drought tolerance or avoidance, and in the droughted or undroughted states.
5. The only observed difference between the species was the greater growth of *Q. palustris* when thoroughly watered.

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The Sensitivity of Several Algae to Ultraviolet Radiation of 2537 Å

By

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Introduction

Numerous workers have shown that algae, in common with other organisms, may be killed or mutated by ultraviolet radiations. There have been relatively few studies, however, on the comparative sensitivity of unicellular algae to ultraviolet radiations. In a recent paper, Holt, Brooks, and Arnold (1951) showed that *Scenedesmus* spp. and *Chlorella pyrenoidosa* had widely different sensitivities to ultraviolet radiation of 2537 Å. The experiments in this study extend these observations to several marine and fresh water algal species at different stages in their growth cycle.

Materials and Methods

Cultures of fresh water and marine algae were used in this study. The fresh water green species used were *Chlorella vulgaris*, *Scenedesmus quadricauda*, *Ankistrodesmus falcatus*, and the yellow-green alga *Botryococcus braunii*. The marine species included the greens, *Chlorella* sp., *Platymonas subcordiformis*, *Dunaliella euchlora* WHOI-1, and the diatoms *Phaeodactylum tricornutum*, and *Skeletonema costatum*. Natural marine populations were collected with a number 20 plankton net from Woods Hole Harbor.

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Fresh-water algal cultures were grown in Rodhe's VIII medium (1948), and the marine species in a modification (McLachlan 1959) of the ASP medium of Provasoli *et al.* (1957). Illumination was provided by fluorescent lights, and the cultures were grown at an intensity of approximately 6,000 meter-candles at 16°C. Culture vessels were 125 ml. Erlenmeyer flasks containing 50 ml. of medium.

Growth was measured by cell counts using a Levy hemocytometer. The results are expressed as the $\log_2 N_t/N_0$, where N_0 is the initial cell concentration and N_t the cell concentration at time t . Photosynthesis was measured by the uptake of $C^{14}O_3$ = according to the procedure of Steemann Nielsen (1952), and pigment analyses were made spectrophotometrically (Yentsch 1957).

A stirred suspension of cells was irradiated in a rectangular lucite chamber (20 ml. volume) with quartz end windows. The chamber was placed eight millimeters from a Westinghouse germicidal lamp (No. 794-H) which emitted nearly monochromatic 2537 Å. The intensity at the end window of the chamber determined with an Eppley thermopile connected to a galvanometer was approximately 136 microwatts per square centimeter. Controls were irradiated in pyrex vessels, which do not transmit 2537 Å, to check for any loss of activity not attributable to the ultraviolet radiation.

To control the absorption of incident energy during growth, suspensions were adjusted to the same cell density prior to irradiation. The cells were then irradiated for one hour with 2537 Å (with a total intensity of eight millijoules per square centimeter). Then the irradiated and control cells were "tagged" with carbon 14, and placed under 6,000 meter-candles of illumination at 16°C for one hour. At the end of this time the suspensions were filtered, dried, and the photosynthetic capacity determined by the amount of radioactivity. The pigments were determined spectrophotometrically. The data are expressed as the relationship between the carbon 14 uptake after irradiation (R_0) relative to the carbon 14 uptake of the non-irradiated suspensions (R).

Results

A histogram, Figure 1, shows the relative sensitivities of several algae to 2537 Å. The species fall into three groups. Diatoms and the natural populations, which were predominantly diatoms, are most sensitive. *Scenedesmus*

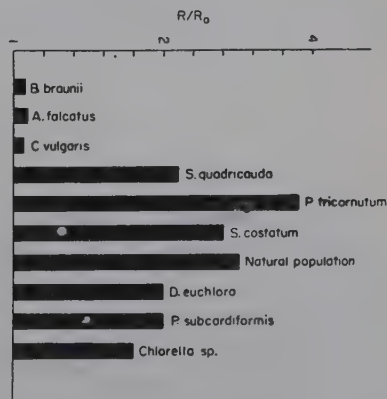


Figure 1. Sensitivity of different algal species to 2537 Å.

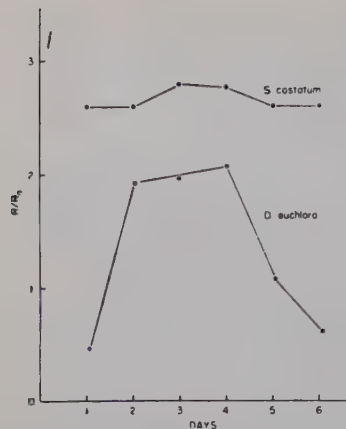


Figure 2.

Figure 2. Relative sensitivity of photosynthesis (R_0/R) of *Dunaliella euchlora* and *Skeletonema costatum* to 2537 Å.

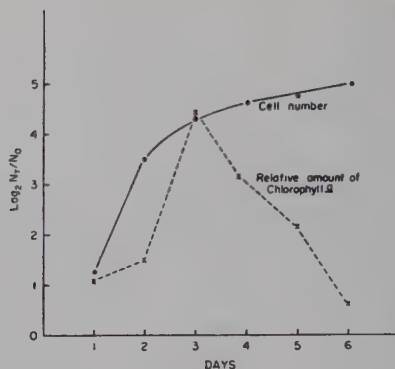


Figure 3.

Figure 3. A comparison of the growth of cells and the change of the chlorophyll a content to 2537 Å in *Dunaliella euchlora*.

quadricauda and the marine green algae were not as sensitive as the diatoms, but were more sensitive than the other fresh water species.

The comparative sensitivity of the algae at different stages in the growth cycle is illustrated for *Skeletonema costatum* and *Dunaliella euchlora* in Figure 2. There is a relatively low sensitivity during the lag phase of growth, followed by a marked increase during the logarithmic phase of growth. The maximum sensitivity is reached at the end of the logarithmic phase, and as the stationary phase progresses there is a gradual decline to the initial level.

Figure 3 also shows that there is a concomitant change in the pigment concentration of *Dunaliella euchlora* during the growth cycle that parallels the impairment of photosynthesis by 2537 Å. This same phenomenon occurred in the other species.

Discussion

It has recently been suggested (Clayton *et al.* 1958) that the carotenoid pigments act as a screen or block to ultraviolet radiation, and that ultraviolet damage is incurred in cells with a low carotenoid content. Diatoms, however, with a relatively high carotenoid content, are more sensitive to 2537 Å than the green algae. Since fucoxanthin efficiently transfers the energy to chlorophyll *a* (Duysens 1952), this implies that the carotenoid screen is only effective

tive if these pigments are not closely associated with the photosynthetic mechanism.

The present results show that algae have widely different sensitivities to 2537 Å radiation. The form of the curve after radiation depends on the stage of growth of the culture. Similar results have been obtained by other workers with bacterial species subjected to ionizing radiations (Stapleton 1952). Since irradiated cells are able to perform most if not all of the normal metabolic functions, these results suggest a specific inhibition. The correct interpretation, however, must rest on a better knowledge of the effect of age, nutritional status, and other environmental factors on the sensitivity of cells exposed to ultraviolet radiation.

Summary

The comparative sensitivity of several algae, during growth, to 2537 Å is as follows: (1) a low sensitivity during the lag phase of growth, followed by an increase during the logarithmic phase of growth, and (2) a decline in sensitivity during the stationary phase of growth. There is a concomitant change in pigment content which parallels the impairment of photosynthesis by 2537 Å.

Appreciation is extended to Dr. B. H. Ketchum and Dr. J. H. Ryther for a critical reading of the manuscript. Supported in part by a grant (G 2510) from the National Science Foundation.

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Inhibitory Effects of 2-Desoxy-D-galactose upon Plant Roots

By

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In previous communications (Stenlid 1954, 1957 a, b, 1959 a, b) the following sugars have been reported as inhibitory to plant roots in different respects: D-mannose, D-galactose, D-glucosamine and 2-desoxy-D-glucose (2-DG). Of these sugars D-galactose takes a special position, inhibiting mainly the growth, whereas it does not reduce respiration and ion absorption. The three other above-mentioned sugars as a rule inhibit growth, respiration and ion absorption more or less distinctly. Structurally all these sugars are very similar to D-glucose. D-galactose deviates from D-glucose at carbon atom 4, the other sugars at carbon atom 2. The differences in stereochemical structure thus seem to correspond to different types of inhibitory action.

It would be of great interest to study the effects of other hexoses differing from D-glucose at carbon 2 or 4 to see if they behave similarly to the four sugars mentioned. Unfortunately such sugars are difficult to obtain in sufficient quantities, and it has only been possible to test one additional sugar, namely 2-desoxy-D-galactose (2-DGal). This sugar deviates from D-glucose both at carbon 2 and 4 and combines the structural modifications of 2-DG and galactose.

For details about materials and methods, see previous communications (Stenlid 1957 a, b, 1959 a, b). In the present investigation, however, the growth experiments with flax roots were performed as described by Åberg (1950, S-test) at 25°C with a growth period of 18 hours. The preparation of 2-DGal was obtained from L. Light & Co. Ltd.

Chloride absorption in excised wheat roots is inhibited by 2-DGal (Table 1). The inhibition is not very pronounced and especially the effect of higher

Table 1. *The effect of 2-desoxy-D-galactose upon absorption of chloride, nitrate and phosphate in excised roots of wheat and cucumber. The values give the absorption expressed as per cent of control without any sugar. In some experiments the reversing effect of glucose and galactose has been studied. The values are means from at last 6 different tubes from 2 different experiments.*

Plant species	Pretreatment of roots in distilled water, hours	Ion determined	2-DGal added, <i>M</i>	Reversing sugar added, <i>M</i>		
				0	Glucose $3 \cdot 10^{-3}$	Galactose $3 \cdot 10^{-3}$
Cucumber	0	Nitrate	10^{-3}	31	—	—
Wheat	0	"	10^{-3}	43	—	—
"	48	Phosphate	10^{-2}	130	—	—
"	0	Chloride	10^{-3}	71	—	—
"	0	"	$3 \cdot 10^{-3}$	71	—	105
"	0	"	10^{-2}	60	80	94

concentrations is relatively weak. The same tendency was observed for 2-DG, although not so distinctly as for 2-DGal (cf. Stenlid 1957 a). The inhibition caused by 2-DGal is relieved both by glucose and galactose.

Oxygen uptake in freshly excised wheat roots was not significantly affected by $3 \cdot 10^{-2}$ *M* 2-DGal, when tested in the same way as mannose and 2-DG (see Stenlid 1954, 1959 b).

With respect to oxygen uptake 2-DGal seems to behave as galactose, with respect to chloride absorption it is intermediate between galactose and 2-DG. In this connection it is interesting to note that neither galactose nor 2-DGal is a suitable substrate for hexokinase (Sols and Crane 1954, Kipnis and Cori 1959).

Nitrate absorption is inhibited both in wheat and in cucumber roots (Table 1). In roots which have been pretreated in aerated distilled water no inhibition of phosphate absorption was obtained (Table 1).

Root growth was inhibited in all species tested, namely wheat, cucumber, flax and cress (Tables 2 to 4; the growth of cucumber roots in 10^{-4} *M* was 42 per cent of the control). The inhibitions of cress and flax roots were re-

Table 2. *The effect of 2-desoxy-D-galactose, alone and in combination with glucose upon the growth of wheat roots. The growth is expressed as per cent of control without any sugar added.*

2-DGal added, <i>M</i>	Glucose added, <i>M</i>				
	0	$3 \cdot 10^{-5}$	10^{-4}	$3 \cdot 10^{-4}$	10^{-3}
10^{-5}	104	—	—	—	—
$3 \cdot 10^{-5}$	86	—	—	—	—
$4 \cdot 10^{-5}$	70	—	—	—	—
$6 \cdot 10^{-5}$	46	—	—	—	—
10^{-4}	31	46	68	90	92

Table 3. *The effect of 2-desoxy-D-galactose, alone and in combination with other sugars upon the growth of flax roots. The growth is expressed as per cent of control without any sugar added.*

2-DGal added, M	Sugar added, M							
	0	2-DG		Galactose		Glucose		
		$3 \cdot 10^{-5}$	10^{-4}	10^{-3}	$3 \cdot 10^{-3}$	$3 \cdot 10^{-4}$	10^{-3}	$3 \cdot 10^{-3}$
0	100	77	26	71	96	—	—	102
$3 \cdot 10^{-5}$	103	—	—	—	—	—	—	—
10^{-4}	58	58	22	—	95	—	—	—
$3 \cdot 10^{-4}$	20	—	—	49	—	72	106	95

versed both by glucose and by galactose, and the inhibition of wheat roots by glucose. In addition to the experiments in Table 2 some experiments with combinations of 2-DGal and other inhibitory sugars have been performed with wheat roots. No distinct signs of a mutual antagonism similar to that between galactose and mannose (see Stenlid 1959 b) were observed.

In flax roots reversal was obtained with galactose, which itself is inhibitory (cf. Stenlid 1959 b). The concentrations of galactose necessary for inhibition are much higher than those of 2-DGal. On the assumption that the sugars inhibit at the same point and compete for the same sites the effects can be explained presuming galactose being able to displace 2-DGal. In combinations of 2-DG and 2-DGal, which are about equally efficient inhibitors, no reversal of the growth inhibition was observed.

The inhibitory effects of 2-DGal upon growth thus mainly agree with those of 2-DG and not with those of galactose. This might be interpreted as an evidence for different types of inhibition of 2-DGal and galactose. It is, however, also possible that the two sugars inhibit growth in the same way, but that only galactose may be converted to glucose in cress and cucumber roots.

The present data about the effects of 2-DGal upon roots are too fragmentary to allow any definite comparison with the other inhibitory sugars. 2-DGal is not acted upon by hexokinase and it does not seem to be phosphorylated in rat diaphragm (Kipnis and Cori 1959). In several respects

Table 4. *The effect of 2-desoxy-D-galactose, alone and in combination with glucose and galactose upon the growth of cress roots. Growth values expressed as per cent of control without any sugar added.*

2-DGal added, M	Reversing sugar added		
	0	Glucose 10^{-3} M	Galactose 10^{-3} M
$3 \cdot 10^{-5}$	91	—	—
10^{-4}	51	106	109
$3 \cdot 10^{-4}$	32	—	99

2-DGal is more reactive than the unmodified D-galactose (Foster *et al.* 1951, Lea and Rhodes 1952) and the conspicuous effects of 2-DGal upon roots show that it in some way influences the metabolism. It is, however, not possible to draw any conclusions about its mode of action, as it is not known if there is any enzyme which can use the sugar as a substrate or which is inhibited by it.

Summary

The effects of 2-desoxy-D-galactose upon plant roots have been studied. The growth of seedling roots of wheat, flax, cress, and cucumber was inhibited. 50 per cent inhibition was obtained with a concentration of about 10^{-4} M. The inhibition is reversed by glucose and galactose in cress and flax roots and by glucose in wheat roots.

In wheat roots chloride and nitrate absorption was inhibited by 10^{-3} M 2-desoxy-D-galactose, whereas oxygen uptake was not significantly affected by $3 \cdot 10^{-2}$ M.

The inhibitory effects are intermediate between those obtained with galactose and 2-desoxy-D-glucose, which is in agreement with the chemical structure of the sugars.

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A Study of the Toxic Effects of Elevated Oxygen Tension on Plants

By

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It is known that variations in oxygen tension alter the sensitivity of organisms to harmful agents. Thus anoxia reduces the lethality of ionizing radiation in some mammals (8, 19) whereas increased oxygen tension has the opposite effect (2, 18). It should be pointed out, however, that elevated oxygen tension in itself may be highly toxic to organisms, as has been demonstrated by several investigators (24), especially in the recent work of Gerschman *et al.* (11) which has sought, among other aims, to provide evidence for a free-radical generating effect of oxygen in biological systems, and to establish a common denominator in the effects of high oxygen pressure and x-radiation on biological systems.

The relation of oxygen to the growth and developmental processes of plants has occupied the attention of several investigators (1, 5, 6, 21, 25, 26), although the question of toxicity has not been a matter of general concern. Oxygen as a general physiologic agent was treated extensively in the classic studies of Paul Bert (4) which encompassed animals, plants, and micro-organisms; and the growth of rice (20) and soybeans (15) as limited by normal atmospheric oxygen tension have been subjects of specific studies. The role of oxygen in root activity was treated from the standpoint of problems of aerations by Clements (7) who reviews the earlier literature extensively.

During a study of the peroxigenic system in plant tissues, Galston and Siegel (10) found that pea root tips were severely damaged when exposed to

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pure oxygen at atmospheric pressure; however, other investigations dealing with oxygen toxicity have dealt mainly with mammals or ciliate protozoans and have not in recent years considered the utility of plants or plant tissues. Notable exceptions include Eliasson's incisive study of wheat root growth inhibitors (9) and the investigation of oxygen-induced inhibition of respiration by Quartley and Turner (21). The present work represents a study of the behavior of the vascular plant on exposure to elevated oxygen tensions with examination of the general characteristics of the oxygen effect and the value of certain protective agents established for animals.

Materials and Methods

Plant Materials: Embryonic axes were excised from air dry seeds of *Phaseolus vulgaris* var. Red Kidney; germination and seedling growth were studies using *Zea mays* var. Stowell's Evergreen. Bean and corn varieties were obtained from commercial stocks of the 1956 crop. For study of responses to acute treatment, most plants employed were obtained from greenhouse stocks at the University of Rochester. A few species, collected near the Genesee River in the vicinity of the campus, included cuttings of *Acer saccharinum*, *Acer megundo*, *Fraxinus* sp., *Parthenocissus quinquefolium*, *Populus deltoides*, *Quercus* spp., *Tilia americana*, *Viburnum acerifolium*, *Vitis riparia*, and gametophytes of all Bryophytes listed below. Collections were made in June and July, 1957.

Pressure Equipment: Acute treatments with oxygen and continuous culture at elevated pO_2 alike were carried out in special heavy walled pressure chambers (Fig. 1). Extreme pressures were obtained using special steel cylinders with a $\frac{3}{4}$ inch wall thickness and threaded cover, and capable of withstanding pressures far in

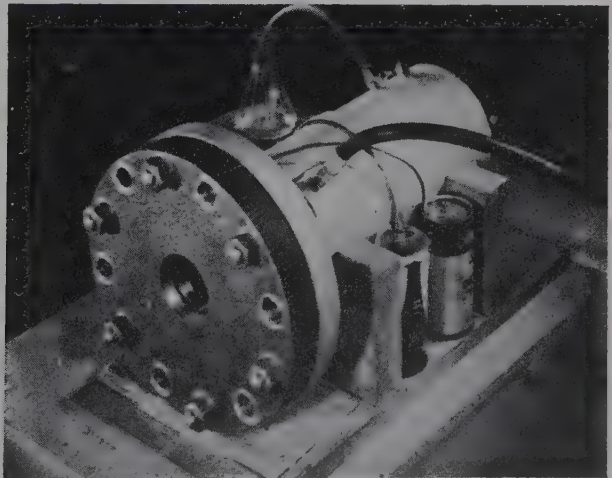


Figure 1. Pressure chamber for acute treatment of mature plants and continuous culture of seeds and seedlings.

excess of the 1800 psi used. Air controls were germinated in 9 or 13 cm petri dishes on filter paper. Germination and growth was carried out at a temperature of $25 \pm 2^\circ\text{C}$, in darkness.

Special Methods: Aside from the ordinary techniques of growth measurement, the activity of the enzyme peroxidase was determined. These assays consisted of photometric determination (at 425 m μ) of pyrogallol oxidation in the presence of hydrogen peroxide, according to detailed accounts already published (28).

Most of the data presented here is expressed in the form of percentage inhibition by oxygen, hence as a ratio. Each of the values employed in calculating the ratio was itself the mean of four to five replicates aggregating 50—60 individuals. The t-Test was used for analysis of the significance of the oxygen inhibition itself and the chi-square Test was applied to the difference between oxygen inhibitions when plants were cultured in water versus experimental solutions.

Results

General characteristics of oxygen damage: The life processes of excised bean embryos are markedly affected when they are incubated in pure oxygen (Fig. 2). Growth seems to proceed to a fair degree at 1 atmosphere pressure, but it should be noted that by the standards of seed viability tests, with the embryo *in situ*, the germination (radicle emergence) percentage in pure oxygen did not exceed 5 %. In oxygen at 2 atmospheres, no germination occurred at all. In contrast, the sample employed contained about 90 % viable seeds after 72 hours incubation by usual germination test in air. The small proportion of seeds germinating in the 1 atm environment are far from normal in growth rate, and cannot maintain even a limited amount of activity for as much as one week after emergence. In addition to elongation of the embryonic axis, and fresh weight increase (*i.e.* water uptake), the formation of the enzyme peroxidase is seen to be quite sensitive to oxygen tension, being approximately 97 % inhibited at 1 atm. When free hand sections of cotyledons from seeds incubated at 2 atm. oxygen were immersed in $\text{I}_2 \cdot \text{KI}$ solution for a starch test, it was found that the color reaction was far deeper than in air-cultured control tissues, indicating that digestion of reserve polysaccharide had been retarded, directly or indirectly. A similar comparative picture was obtained from embryonic hypocotyls.

Application of extreme oxygen pressures as a pretreatment to air dry embryos resulted in effects on growth during subsequent culture in air. For example, embryos exposed to 120 atm. pressure for 3 hrs. showed only a slight inhibition of elongation and water uptake, but a marked reduction in root hair and adventitious root development. Of control embryos tested, 95 ± 5 % exhibited root primordia along the basal half of the hypocotyl surface, but of those exposed to high oxygen pressure, only half developed any root primordia at all. Among the controls with root primordia a mean value of 5.0 ± 0.1 per embryo was observed, whereas the correspond-

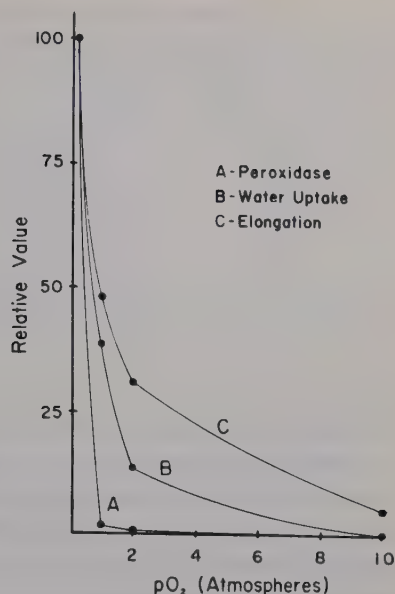


Figure 2. Growth and peroxidase activity in bean embryos cultured for three days at several oxygen tensions.

ing value for oxygen-injured embryos had fallen to 3.8 ± 0.5 ; the difference between control and treated means is significant at the 5 % level. Embryos allowed to imbibe water for one hour were killed by exposure for 30 min. to 120 atm. oxygen pressure. When argon was substituted for oxygen, a slight, non-significant stimulation of elongation was noted during culture after 3 hrs. at 120 atm., and no other sign of an effect of pressure alone observed.

Acute treatment of mature plants: Growth studies were augmented by observations of immediate growth-independent effects of elevated oxygen tensions on a series of mature plants. For preliminary studies, a standard pressure of 6 atm. was adopted, and a group of selected species exposed to this pressure for varying time periods. Represented were 22 species of the Angiosperm genera: *Acer*, *Alyssum*, *Begonia*, *Bryophyllum*, *Coleus*, *Chrysanthemum*, *Erigeron*, *Euphorbia*, *Fraxinus*, *Mesembryanthemum*, *Opuntia*, *Parthenocissus*, *Pelargonium*, *Polygonum*, *Populus*, *Quercus*, *Tilia*, *Viburnum*, and *Vitis*. Pteridophytes were represented by *Cyrtomium*, *Nephrolepis*, *Dryopteris*, *Polypodium*, and *Pteris*. The few Bryophytes tested were of the genera *Funaria*, *Marchantia*, *Polytrichum*, and *Rhodobryum*.

Slight effects of 2 to 3 hours exposures were observed with increasingly severe damage accompanying prolongation of treatment times up to 15 hours, which was lethal for most of the plants tested. Longer exposure times hastened the appearance of injury signs after removal of plants from pressure chambers. Even after 45 hours exposure, treated plants were of essentially

Table 1. *Effects of CoCl₂ on bean embryo growth in oxygen.*

Growth process	Medium	Percentage inhibition in oxygen	
		pO ₂ = 1.0	pO ₂ = 2.0
Elongation	Water 10 ⁻⁴ M CoCl ₂	52 0 ²	60 7 ²
Water uptake	Water 10 ⁻⁴ M CoCl ₂	80 40 ¹	90 65 ¹

¹ Difference from water control significant (P=0.95).

² Difference from water control highly significant (P=0.99).

normal appearance, save for some browning of leaf margins and darkening of chlorophyllous areas, and an additional 3 to 24 hours was required for lethal effects to be manifested. In general, 12 to 24 hours after termination of a 15 to 20 hour treatment, discolorations increase, if already present, or appear; leaves desiccate (usually, the more mature leaves first), becoming darkened and shrivelled; stems, if not rich in mechanical tissue, collapse and shrivel; petioles shrivel and in some cases, *Begonia* and *Euphorbia*, for example, absciss; succulent leaves develop patches of discoloration which spread, become moist and finally desiccate as in the case of *Bryophyllum* and *Begonia*; apical regions of stems often become recumbent. Additional, specific, observations which are of value in assessing the nature of oxygen damage include: (a) discolorations in *Coleus*, young green leaves of red varieties turning red, and older, anthocyanin-bearing leaves becoming brown; (b) exudate droplets on the succulent leaves of *Mesembryanthemum*, which were found to contain sugars, starch, and protein, including the enzyme peroxidase (molecular weight, about 50,000); and (c) the resistance of yellow *Chrysanthemum* petals to oxygen treatments causing discoloration and complete desiccation of ordinary green leaves.

Among the plants tested, the fern species were most resistant to elevated oxygen tensions. Although delayed effects upon growth were observed 5 to 7 days after treatment, only a slight browning of pinnule margins was noted as an immediate after-effect of exposure to 6 atmospheres for 15 hours. Even after exposure to 10 atm. for 15 hours, the fern fronds retained sizeable areas of normally colored, unwilted tissue, unlike the Angiosperms, all of which were killed by this more severe treatment. The Bryophytes tested lacked features permitting a ready evaluation of their susceptibility to high oxygen tensions, but, like the ferns, seemed to be more resistant than the flowering plants. Considering at the organismal level, the plants treated above, "recovery" was expressed as the stimulation of axillary bud development, so

Table 2. *Protection of corn against elevated oxygen tension.*

Growth measurement	Inhibition by 2 atm. oxygen (% of air control) in:		
	Water	10^{-5} M CoCl_2	10^{-5} M cysteine
Root Emergence	21	0	0
Root Elongation	75	53 ²	46 ²
Root Hairs	81	60 ²	30 ²
Coleoptile Emergence	28	20	20
Coleoptile Elongation	59	5 ¹	58

¹ Difference from water control significant ($P=0.95$).

² Difference from water control highly significant ($P=0.99$).

that a plant whose stem apex and emerged leaves had been entirely killed by oxygen treatment could nevertheless survive as an individual. Of interest in this connection was the behavior of *Pelargonium*, the new, axillary shoots of which bore a somewhat greater proportion of malformed albino leaves than were found among the normally variegated population of control plants. Thus, it is possible that long-term physiological effects may be observable in the more protected tissues.

Protection against oxygen damage: When bean embryos were cultured in 10^{-4} M CoCl_2 , their susceptibility to injury during exposure to pure oxygen at 1—2 atm. was markedly reduced (Table 1). Growth, as measured both by elongation and water uptake over a 50 hour incubation period, was much less inhibited in the presence of the cobaltous ion. In some cases, protection was complete, or nearly so; a definite protective effect was in evidence both at 1 and 2 atm. for elongation and water uptake alike.

Further experiments on oxygen damage and protection therefrom were carried out with corn. Only germination and early seedling development are considered here, although effects of oxygen treatments during germination have been observed during subsequent growth and development for at least 30 days. Corn grains were incubated in air for 48 hours, in water, or in solutions of CoCl_2 or cysteine, rinsed with distilled water, and transferred to sterile petri dishes containing filter paper moistened with water only for exposure to oxygen. Although the effects of cobaltous ion or cysteine concentration have not been studied thoroughly, concentrations in excess of 10^{-5} M, especially of the thiol, tended to be toxic in themselves.

As a result of the two-day air incubation, it is not surprising to find little inhibition of germination (root emergence) on incubation of corn grains at 2 atm. oxygen pressure (Table 2). Coleoptile emergence is similarly little affected, but other growth processes measured were markedly retarded in oxygen. Both cobaltous ions and cysteine can protect root and root hair

growth against the effects of elevated oxygen tension, however only the former substance protects the coleoptile, no effect of the thiol being in evidence. In contrast to its lack of effectiveness in relieving oxygen-inhibition of coleoptilar growth, cysteine is somewhat more effective than cobaltous ion in root elongation and markedly more effective than cobaltous ion in root hair development.

Other experiments which have been carried out, using ultraviolet or x-radiation instead of elevated oxygen tension present an essentially similar picture relative to kinds of damage manifested and the protection by CoCl_2 or cysteine. Dosages on the order of 10^5 r (x-radiation) or 10^7 erg.mm.⁻² (2537Å) which were required for severe damage to plant tissues or organs could be at least partially offset by the presence of these protectants. Detailed studies of radiation effects will be treated separately.

Discussion

The present work is concerned mainly with the demonstration that a toxic oxygen effect of considerable magnitude exists, and that the severity of damage may be effectively controlled by simple, exogenously supplied chemical agents. The protective activity first demonstrated for cobalt (II) in mice (13), paramecia (14), and *in vitro* systems (16) and for cysteine in mice (11) is here shown to be phenomenon of general significance, applicable to plants in various stages of development.

The same reasoning involved in previously cited proposals of oxidizing radical mechanisms is fully valid with plant material when the similar action of the same protectants against oxygen, x- and ultraviolet radiation are taken altogether. Further, antioxidants reduce the damage resulting from exposures of plant tissues to high temperature shock treatments, and the magnitude of heat damage was far smaller when elevated temperatures were applied to oxygen-free material (23). Additional comparison is provided by the responses of bean embryos to 120 atm. oxygen pressure. This treatment characteristically reduced root hair and adventitious root development without significantly affecting elongation of the embryonic axis. Embryos exposed to x- or ultraviolet radiation, or to high temperatures also show impairment of root and root hair production at dosages unable to reduce elongation. For example, root and root hair formation is prevented by an x-ray dosage of 50,000 r, whereas elongation of the embryonic axis remains uninhibited even after an x-ray treatment of 150,000 r (Siegel, unpublished observation).

Although the several agents considered may also exhibit qualitatively distinct, *i.e.* specific, effects, the proposition that a common chemical mechanism underlies all of them assuredly merits consideration.

If it be accepted provisionally that a diversity of "activating" agents share in large measure a common pathway of action, then it may be further noted that the effects of heat shock treatments seem to have much in common with an accelerated aging process, and that the fundamental histo-pathology of mammals suffering from chronic exposure to ionizing radiation is also that of an accelerated but otherwise typical senile degeneration (6, 17, 22). Considering finally the common occurrence of uncompensated oxidations in differentiating or senescent tissues, in melanization and lignification for example, it is reasonable to propose that oxygen and oxygen-activating agents of various sorts contribute substantially to the normal aging process; the accelerated rate of deterioration noted under experimental conditions is, then a reflection of the heightened degree of activation of oxidative processes.

Although the array of specific responses to elevated oxygen tension described in this communication are by no means unique to aging or senescent plant tissues, they are, nevertheless, entirely consistent with the concept of aging processes accelerated by unregulated and unbalanced oxidative changes (12).

Summary

Excised bean embryos cultured in pure oxygen show marked reductions in growth and enzyme activity, even at normal atmospheric pressure. Root and root-hair development were retarded by exposure of dry embryos to 120 atm. for 3 hrs. before culture; elongation of the main axis was not affected.

Mature plants (31 species from 28 genera of Angiosperms, Pteridophytes and Bryophytes) were tested for growth-independent acute effects at 6 atm. oxygen. Exposure for 15 hrs. was generally lethal, but signs of damage were delayed in appearance as much as 24 hrs. after ending treatment. Responses include: discoloration; desiccation and withering of leaves; stem collapse; abscission (*Begonia* and *Euphorbia* especially); and recumbent stem apex. *Mesembryanthemum* leaves bore droplets of exudate containing proteins and polysaccharides.

Ferns are highly resistant, tolerating an oxygen tension of 10 atm., lethal to all Angiosperm species tested.

Bean embryos were protected against oxygen damage by culture in 10^{-4} M Co(II) as measured by elongation and water uptake. Root hair development and root elongation of corn seedlings were greatly inhibited by 2 atm. oxygen, but Co(II) or cysteine at 10^{-5} M reduced inhibition substantially.

Protection by Co(II) of pea root tips against x-radiation was compared with its similar effect in the case of oxygen poisoning. The protective effect of Co(II) and cysteine against ultraviolet radiation damage is also noted.

The general biological significance of these phenomena are pointed out, especially the parallels which may be drawn between characteristics of oxygen, radiation, and thermal damage and senescent deterioration.

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Rôle of Cellular Ribonucleic Acid in the Growth Response of *Avena* Coleoptile to Auxin

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The present author reported that auxin influences the permeability of plant cells to water and to non-electrolytes (12, 14). He also demonstrated that auxin reduces the exchangeability with extracellular K ion of the Na previously accumulated in cells of *Avena* coleoptile (15) and suggested that the capacity of cation binding in the cells is affected by auxin.

On the other hand Hiraoka *et al.* (8) and Takada (21) found that the uptake of both basic dyes and inorganic cations by yeast cells is markedly reduced by the pretreatment with ribonuclease. They conclude that chiefly cellular ribonucleic acid is involved in the cation binding of cells. Similar facts were also reported by Tanada in rubidium uptake by mung bean roots (22, 23, 24).

In the third place, the loosening of the cell wall is considered as the primary growth promoting effect of auxin according to Tagawa *et al.* (19), Cooil *et al.* (5), and others.

When these three accounts are brought together, a hypothesis is formed that the ribonucleic acid acting as a cation binder at or near the protoplasmic surface is involved in the reaction chain to be initiated by auxin and to result in the loosening, and hence the elongation, of the cell wall. The present paper reports the results of some experiments performed to test this hypothesis.

Material and Methods

The Avena (Victory I) seeds were first soaked in distilled water for 3 hours and germinated at 25°C on filter paper moistened with distilled water in Petri dishes. Seedlings were set in vermiculite in glass trays after 24 hours, and kept for 68—70 hours under 90 % relative humidity at about 25°C. Seedlings having about 3 cm. long coleoptiles were then selected and decapitated. Two hours thereafter, the primary leaf and 2 mm. of the apical region was discarded, and one 4 mm. or 8 mm. long section from each coleoptile was cut out with a double-bladed cutting tool. Thus made sections were divided into lots of 10 and floated on 10 ml. of the test solutions in beakers. They were incubated at 25°C in the dark and taken out of the solution at intervals of 30 or 60 minutes for length measurements under 20 power magnification with an ocular micrometer. The light was dim enough to avoid interference with the growth (2, 4).

Ribonuclease (RNase) was dissolved in 1/100 *M* phosphate buffer solution (pH 7.0). The solution was heated at 70°C for 20 minutes before use to inactivate contaminating proteinase (17). The sections were treated with RNase by floating on the solution at 30°C for 60 minutes.

The uptake and the efflux of pyronine B (PB) was measured by floating 30 of 4 mm. long sections or 15 of 8 mm. long ones on 3 ml. of 1×10^{-5} *M* solution of PB and measuring the absorbancy at 554 mμ of the external solution at intervals. It was assumed that these measurements gave informations about the cation binding capacity of cellular ribonucleic acid (RNA).

To say generally, the growth increment differences greater than 0.03 mm. and the differences of PB uptake (extinction) greater than ($E =$) 0.02 are significant at 5 % level.

Results

A. Effect of ribonuclease on the PB uptake and on the auxin-induced elongation

In Figure 1 are shown the processes of the elongations as well as of the changing elongation rate (mm./hour) of the coleoptile sections in 1 mg./l. indoleacetic acid (IAA) solution, which has been determined to be markedly effective in long term observations (12—24 hours) (3, 13). Elongation rate reaches its maximum within the first 60 minutes, to decrease thereafter. This fact is consistent with the results by Busse *et al.* (4) and Marinos (11). It seems certain that the primary elongation effect of auxin appears within 60 minutes in the sections.

In order to see the effect of IAA pretreatment on the PB uptake the sections were floated for 30 minutes on 1 mg./l. IAA solution, or on distilled water as control, and transferred to 1×10^{-5} *M* PB. As shown in Figure 2, PB was absorbed considerably within the first 5 minutes, and gradually then. The PB uptake was reduced by the IAA pretreatment, especially during the

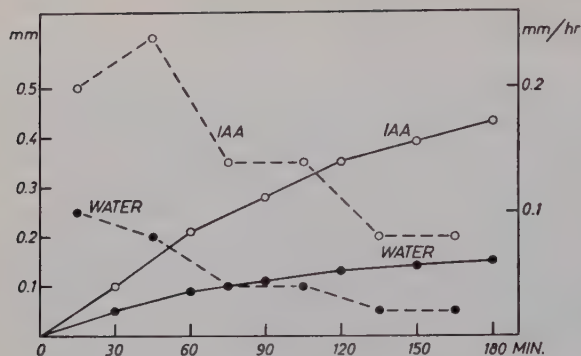


Figure 1. Time course of elongation (mm.) (—) and rate of elongation (mm./hr.) (---) of the coleoptile sections with (○) and without (●) 1 mg./l. IAA given to at zero time.

first 5 minutes. It seems, therefore, that the binding capacity of the RNA at the protoplasmic surface, which may relate to the permeability, is roughly reflected by this initial PB uptake. Hence the PB uptake in the initial 5 minutes was measured in the following experiments.

As shown in Figure 3 the reduction of PB uptake due to the IAA pretreatment was at its maximum after 30 minutes. The greater PB uptake by the 4 mm. sections than by the 8 mm. sections indicates that the cut ends of the section take up a considerable amount of PB. According to calculations, using the data of 4 and 8 mm. sections, the PB uptake at the cut ends is not affected by IAA while that at the intact part is reduced by IAA to about 30 % of control.

Effect of pretreatments with other auxins and related compounds on the PB uptake are shown in Table 1. All the three auxins employed reduced the uptake, but none of the others, which induced no elongation within 180 minutes, was effective. The results are in favour of the view that the reduction of PB uptake has substantial connection with the physiological effect of auxin.

Effect of the RNase treatment on the PB uptake is shown in Table 2. According to calculations based on the data obtained, the PB uptake at the cut ends is reduced by the RNase treatment to half and that at the intact part to about 70 %. The effect of RNase on the PB uptake by living cells has also been observed with yeast cells (21). The sections pretreated with RNase did not respond to IAA for about 90 minutes, but appeared to restore the reactivity gradually thereafter (Figure 4). The sections pretreated with RNase recovered the capacity of PB uptake in parallel with the recovery of the elongation effect of auxin (Table 3).

It is represented in Table 5 that the commercial yeast RNA promotes elongation of the sections, although Na salt of RNA does not (17). If, how-

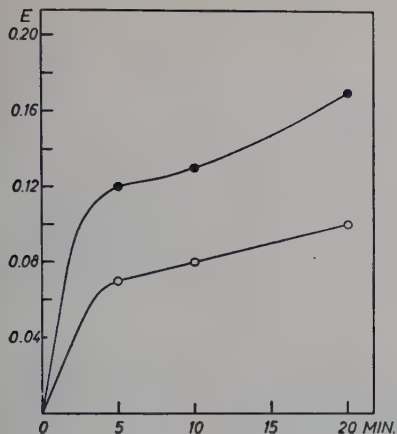


Figure 2.

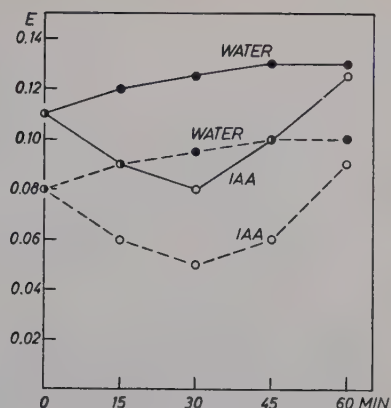


Figure 3.

Figure 2. Time course of PB uptake (E) by 30 sections of 4 mm. length pretreated (○) and not pretreated (●) with IAA for 30 minutes.

Figure 3. PB uptake (E) by the sections pretreated (○) and not pretreated (●) with IAA for various periods (on the abscissa). —: 30 sections of 4 mm. length, ---: 15 of 8 mm.

ever, the sections were pretreated with RNase, the yeast RNA did not apparently promote the elongation until about 90 minutes (Figure 4) just as with IAA. It seems that the yeast RNA can promote the growth of the sections only when intact RNA exists at the protoplasmic surface.

When the sections were treated with relatively concentrated salt solutions, washed with distilled water, and immersed in PB solution, the dye uptake was lowered (Table 4). Similar results have been reported for yeast cells (20, 8).

Table 1. PB uptake by 15 sections of 8 mm. length pretreated with auxins and non-auxin substances for 30 minutes.

Pretreatment (30 min)	PB uptake (Extinction)
Water	0.09 ± 0.00
IAA (1 mg/l)	0.06 ± 0.00
2,4-D (1 mg/l)	0.06 ± 0.01
NAA (1 mg/l)	0.06 ± 0.00
Tryptophan (5×10^{-6} M)	0.09 ± 0.00
Isatin (5×10^{-6} M)	0.10 ± 0.00
Acetic acid (5×10^{-6} M)	0.09 ± 0.00
Acetic acid (5×10^{-5} M)	0.09 ± 0.00

Table 2. Effect of RNase pretreatment on the PB uptake by the sections, 30 of 4 mm. ones and 15 of 8 mm.

Pretreatment (60 min)	PB uptake (E)
4 mm. sections {buffer only	0.13 ± 0.01
{RNase ...	0.08 ± 0.00
8 mm. sections {buffer only	0.10 ± 0.00
{RNase ...	0.07 ± 0.00

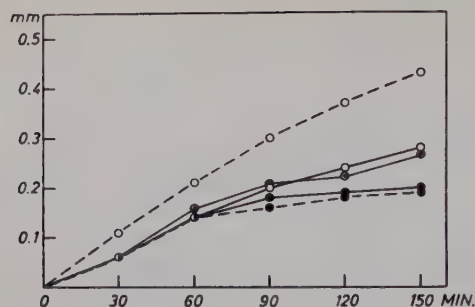


Figure 4. Effect of RNase pretreatment on the elongation in water (●), IAA (○) and 0.1 % yeast ribonucleic acid solution (⊙). ----: pretreated with phosphate buffer solution, —: pretreated with RNase.

The above-mentioned results together lead to the assumption that the state of cellular RNA capable of binding PB is a prerequisite of the initial growth reaction to be affected by auxin, the site of PB binding being capable of binding inorganic cations.

B. Effect of Ca-complexing agents on the elongation of the coleoptile sections

Tagawa *et al.* (19) and Cooil *et al.* (5) infer that the plasticity of cell wall increases when calcium ions are removed from Ca-pectate in it. Associating this account with the idea presented above a hypothesis is led that IAA, through some mechanism, influences cellular RNA and increase its capacity of Ca binding, to result in a decrease of Ca in the cell wall.

If Ca is removed from the cell wall by some means, an increased elongation of the sections will occur even without exogenous supply of IAA. The effect of disodium ethylenediaminetetraacetate (EDTA) on the elongation

Table 3. Effect of RNase treatment followed by IAA and OXA treatment on the PB uptake by 30 sections of 4 mm. length.

1st treatment (60 min)	2nd treatment	PB uptake (E)
Buffer soln.	none	0.13 ± 0.01
RNase	none	0.08 ± 0.00
RNase	water 30 min	0.09 ± 0.00
	water 120 min	0.12 ± 0.00
RNase	IAA 30 min	0.09 ± 0.00
	IAA 120 min	0.10 ± 0.01
RNase	OXA 30 min	0.08 ± 0.00
	OXA 120 min	0.12 ± 0.01

Table 4. PB uptake by 15 sections of 8 mm. length pretreated with $1/10$ M NaCl and $1/15$ M CaCl_2 solutions. The values denote PB uptake (E).

Pretreatment	Periods of pretreatment	
	30 min	60 min
Water	0.10 ± 0.01	0.11 ± 0.01
NaCl	0.07 ± 0.00	0.05 ± 0.00
CaCl_2	0.07 ± 0.01	0.05 ± 0.00

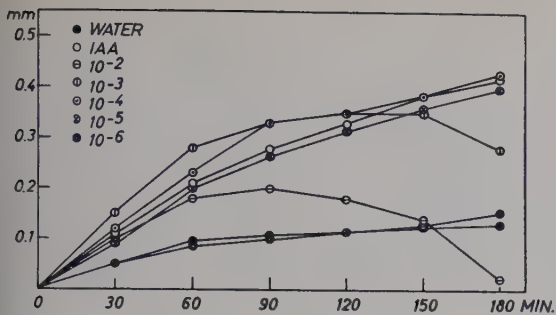


Figure 5.

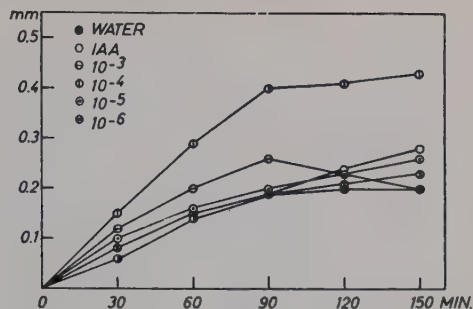


Figure 6.

Figure 5. *Effect of oxalic acid. Concentrations in M indicated on the curves.*

Figure 6. *Effect of oxalic acid on the RNase-pretreated sections. Concentrations in M indicated on the curves.*

of coleoptile sections is shown in Table 5. 8-Hydroxyquinoline at 10^{-5} M promoted the elongation to about same extent as IAA for the initial 60 minutes (Table 5). These facts are consistent with the results by Bennet-Clark (1) and Heath *et al.* (6, 7). Agents which form water-insoluble salts with Ca ions, such as phosphotungstate, ammonium molybdate, ammonium phosphate and ammonium sulfate, also promoted the elongation of the sections (Table 5).

In the case of oxalic acid (OXA), 10^{-4} M was more effective than IAA within 180 minutes, as shown in Figure 5. It was at 10^{-3} M even more effective than 10^{-4} M at first, though the sections later became shrunken. Even when the sections were made non-reactive to IAA by the RNase pretreatment, OXA promoted the elongation (Figure 6).

EDTA and OXA have an effect of restoring the PB uptake by the sections which are pretreated with IAA for 30 minutes (Table 6). This may mean that

Table 5. *Effect of Ca-complexing agents on the elongation of the sections.*

Agents	Optimal concentration	Elongation (mm)	
		After 60 min	120 min
Water	—	0.10	0.14
IAA	1 mg/l	0.21	0.35
Yeast ribonucleic acid	0.1 %	0.17	0.27
EDTA	10^{-3} M	0.17	0.31
8-hydroxyquinoline	10^{-5} M	0.18	0.23
Phosphotungstate	10^{-5} M	0.16	0.27
Ammonium molybdate	10^{-4} M	0.17	0.28
Ammonium phosphate	10^{-4} M	0.13	0.18
Ammonium sulfate	10^{-3} M	0.15	0.22

Table 6. *Effect of EDTA and OXA on the PB uptake by 30 sections of 4 mm. length pretreated and not pretreated with IAA.*

1st treatment (30 min)	2nd treatment (15 min)	PB uptake (E)
Water	water	0.13 ± 0.01
"	EDTA ($10^{-3} M$)	0.14 ± 0.01
"	OXA ($10^{-4} M$)	0.12 ± 0.00
IAA	water	0.09 ± 0.00
"	EDTA	0.11 ± 0.01
"	OXA	0.10 ± 0.00

Table 7. *Effect of IAA on the PB uptake by 15 sections of 8 mm. length pretreated and not pretreated with OXA solution.*

1st treatment (30 min)	2nd treatment (30 min)	PB uptake (E)
Water	water	0.09 ± 0.00
"	IAA	0.06 ± 0.00
OXA ($10^{-4} M$)	water	0.08 ± 0.01
"	IAA	0.09 ± 0.00

Ca ions are removed by EDTA and OXA from the cellular RNA which has bound Ca ions due to the effect of IAA.

Table 7 represents the PB uptake by the sections which were treated first with OXA or distilled water for 60 minutes and then with IAA or distilled water for 30 minutes. IAA did not reduce the PB uptake when the sections were pretreated with OXA. The most plausible explanation for these results is that *the affinity of PB to RNA is decreased by the formation of a Ca-RNA complex which is enhanced by the IAA pretreatment, and that such an interference by IAA does not occur when Ca has previously been removed.*

C. Effect of PB pretreatment on the subsequent action of IAA

If the above-mentioned hypothesis is true the following is expected: When the binding site of cellular RNA is preoccupied by PB, the elongation of the sections will not be induced by IAA if the RNA-PB binding is strong, and the

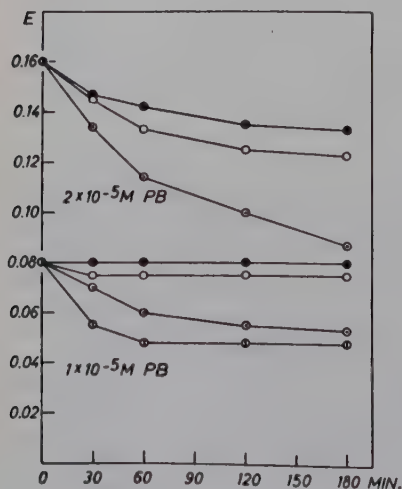


Figure 7. *PB efflux (E) in IAA (○-○), RNase (◐-◐) and 1/15 M CaCl₂ solutions (●-●) by the sections (15 of 18 mm. length) pretreated with 1× and 2×10⁻⁵ M PB solutions.*

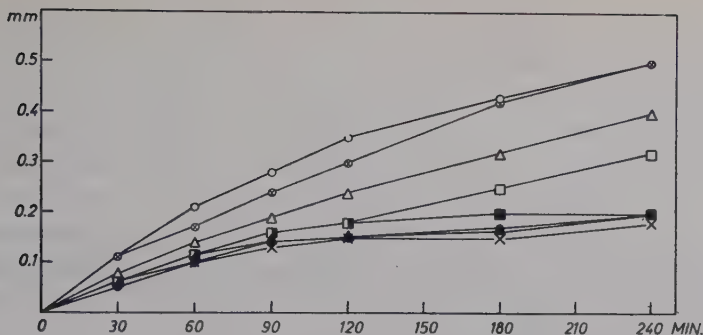


Figure 8. *Effect of IAA on the elongation in mm. of the sections (4 mm. length) pretreated with various concentrations of PB. ○-○: in IAA without PB pretreatment, ●-●: in distilled water without PB pretreatment, ⊗, △, ⊠: in IAA after treatment with PB of 1×10^{-5} , 2×10^{-5} and 3×10^{-5} M respectively, ×, ▲, ■: in distilled water after treatment with PB of the same concentrations.*

PB efflux will occur if the bound PB is replaceable by Ca ions, namely if the elongation is induced by IAA.

The sections were soaked in 1×10^{-5} and 2×10^{-5} M PB solutions for 5 minutes, quickly washed with distilled water, and transferred to IAA, RNase and $1/15$ M CaCl_2 solutions. The efflux in these solutions and in water is represented in Figure 7. PB efflux was enhanced by Ca solution and RNase, but not so much by IAA.

On the other hand, the effect of IAA on the elongation of the sections pretreated with PB is, as shown in Figure 8, very slight at first, but becomes apparent later. The higher the PB concentration, the longer the refractory period. The relation between the two effects of IAA, namely the PB efflux and the elongation, may be explainable by the present hypothesis as such in the earlier part of the elongation effect, while for the later part an additional assumption is needed that the PB efflux is small since the PB bound by RNA is transported to vacuole (cf. 18). The period of 90 minutes is already known as the period necessary for the regeneration of the nature of RNA to bind PB and inorganic cations, and to make use of the exogenous yeast RNA (cf. Figure 4).

Discussion

As reviewed lately by Bentley (2), a number of recent authors on the initial stage of elongation lay stress on the increase in extensibility of the cell wall to be caused by the removal of Ca from pectic substance in it. However,

as to how such removal of Ca is brought about by auxin, no adequate explanation has yet been presented.

According to the studies by Masuda on the *Avena* coleoptile cultured in NaCl solution (15) and by Kawahara *et al.* (9) on *Vallisneria* leaves containing much Na, the efflux of cellular Na and its exchangeability with extracellular K ions are markedly suppressed by IAA. They consider that IAA has an effect on the cellular site of cation binding. That RNA is involved in the cation binding site is pointed out by Takada (21) and Hiraoka *et al.* (8) for yeast cells and by Tanada (22, 23, 24) for mung bean root cells or by Lansing *et al.* (10) for *Elodea* cells.

Experiments presented in this paper are the best explicable by assuming that IAA increases the cation binding capacity of the RNA at the protoplasmic surface of coleoptile cells, so far as it is represented by the primary PB uptake. This account may present a link between auxin and the removal of Ca from pectin in the cell wall.

Then, in what way does auxin increase the binding capacity of cellular RNA? Tanada (22, 23, 24) considers that more sites of RNA for cation binding become available when the nucleic acid moiety in ribonucleoprotein becomes separated from protein. Auxin is known to form a complex with protein in cytoplasm (25). Hence if IAA renders RNA separated from protein by complexing with the latter an increase in cation binding sites may be expected. It is necessary, however, to investigate the relation between auxin and cellular RNA in particular.

When the RNA at the protoplasmic surface undergoes a change in its ion binding capacity, the physico-chemical properties may also be changed. Judging from the plasmolysis form, adhesion between protoplasm and cell wall is increased remarkably by a brief treatment with auxin (16). This may be explained by an increase of Ca binding of RNA at the protoplasmic surface.

The efflux of the K ion from epidermal cells of *Allium cepa* (16) and that of the Na ion from *Vallisneria* leaves (9) are increased by IAA, only when the cells are in the plasmolyzed state. Auxin increases protoplasmic permeability to non-electrolytes such as urea, as measured by the plasmolytic method (cf. 12). It is yet unknown at present, however, whether this change in the plasmolysis permeability depends on a change in cellular nucleoprotein, or it is due to some other secondary effect of plasmolysis. However, since RNase pretreatment makes the plasmolysis form convex, the physico-chemical state of protoplasmic surface appears to be influenced by the ion binding capacity of the RNA at the surface of protoplasm (Masuda, unpublished data).

Thus the mechanism of auxin-induced elongation at its initial stage is

explainable by a change in the ion binding capacity of RNA at the protoplasmic surface. The secondary elongation in which the synthesis of cell wall material must be involved is another problem.

Summary

A hypothesis is proposed to explain the primary cell elongation caused by auxin: Auxin increases the cation binding capacity of ribonucleic acid at the protoplasmic surface, and this in turn takes up calcium from the pectic substance which constitutes the cell wall, which is thus loosened. Experimental results using *Avena* coleoptile sections are favourable to or explicable by this hypothesis;

(1) Following the indoleacetic acid treatment the maximum of the rate of the elongation and the minimum of the pyronine B uptake appear at the period corresponding to each other.

(2) This decrease of the pyromine B uptake is caused also by 2,4-dichlorophenoxyacetic acid and naphthaleneacetic acid, as well as indoleacetic acid, but not by the non-auxin substances used.

(3) When the sections are pretreated with ribonuclease, their pyronine B uptake decreases remarkably. This decrease recovers in 120 minutes when the sections are floated on distilled water.

(4) When the sections are pretreated with ribonuclease, indoleacetic acid does not affect their elongation within 90 minutes, but the elongation effect appears later attended by the recovery of the basophilic capacity of the cells.

(5) Floating on 0.1 % yeast ribonucleic acid solution promotes the elongation, but not when the sections are pretreated with ribonuclease. In the latter case the effect of yeast ribonucleic acid begins to appear after 90 minutes.

(6) When indoleacetic acid is given to the sections which have taken up pyronine B previously, both the pyronine B efflux and the growth promotion are limited. After 90 minutes, however, the indoleacetic acid-induced elongation occurs.

(7) Chelating agents such as disodium ethylenediaminetetraacetate and 8-hydroxyquinoline and Ca precipitants such as tungstate, molybdate, phosphate, sulfate and oxalic acid promote the elongation, and it is effective even when the sections are pretreated with ribonuclease.

(8) The pyronine B uptake is not lowered by indoleacetic acid when the sections are pretreated with oxalic acid.

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Investigations on the Mechanism of Absorption and Accumulation of Salts, IV.

Synergistic and Antagonistic Effects of Anions

By

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Introduction

The laboratory of the author devoted earlier work to the antagonism between metallic cations and between H-ions and metallic cations (see Lundegårdh 1932, 1957, Burström 1934) and the phenomenon has been since then extensively studied in other laboratories, too (cf. Fischer 1956). It is commonly interpreted as an effect of ion exchange, cations being "adsorbed", *viz.* exchangeably attached to large organic ions, which are present as structural elements of the protoplasm and its membranes. Mutual effects may, however, also arise from metabolic interactions. It was shown at an early date that in their exchange relations with the protoplasm cations behave much in the same way as towards inorganic negatively charged colloids, *e.g.* soil colloids and ion exchange resins (Lundegårdh 1932, 1957). Ion exchange may be studied on changes in the surface potential of roots, too (Lundegårdh 1941). The antagonistic phenomena are of course also reflected in the distribution of cations in the bulk of the plant tissues.

Corresponding phenomena regarding anions have been less extensively studied. Observations of changes in the surface potentials under the influence of salts revealed phenomena which could be explained only by the assumption of an adsorption of anions to positively charged counter ions (R^+ ; "positive carriers"). The strong initial absorption may be interpreted as a repeated

exchange of anions in the bulk of the cytoplasm and a non-metabolic gliding along "adsorption tracks" (Lundegårdh 1958).

As to the chemical properties of the loci of salt adsorption little is exactly known. Because *all large organic ions which dissociate H^+ and OH^- may serve as potential "carriers"* it must be concluded that the bulk of the cytoplasm acts as a bilateral adsorbens for cations and anions, *viz.* salts. The recent investigations of Kling (1958) on transport tracks in the cytoplasm ought to be mentioned here. It is a well-known fact that all artificial ion exchange substances show a certain *selectivity* as to adsorbed ions. Examples of specific adsorption of cations in the living substance have also been known for a long time.

Experimental Results

The experiments presented in this communication are focussed on the occurrence of mutual effects of anions during the initial absorption and continued accumulation of salts. The experimental technique is the same as in the previous communications of this series. As material served roots of spring wheat which were pretreated in *aqua destillata* for 48 hours, and thoroughly washed (24 h.) slices of potato tissue. All experiments were conducted at 20°C. Chloride and phosphate were analytically determined by means of previously mentioned conventional methods, and nitrate by means of phenol-disulphonic acid (Burström 1942).

A series of special experiments showed only *inconsiderable differences in the absorption of anions* (including phosphate) *between pH 5 and 8*. The uptake of anions was calculated from the deficit in the medium, because some of the salts, primarily nitrate, are rapidly assimilated during the process of accumulation. Phosphate is assimilated only to a few percent and the uptake may here be controlled by extraction of the tissues. Chloride is partially fixed in the tissues and it is difficult to extract the last traces of this ion.

A number of series were performed with two salts containing the same cation, primarily potassium, and different anions. The following combinations were studied: Cl and H_2PO_4 , Cl and NO_3 , Cl and SO_4 , H_2PO_4 and NO_3 . Each combination was tested in two series. One of the two anions was held in a constant concentration, mostly 0.005 M, and the quantity of the other anion was varied from zero to 0.020 M. 2 g. of material was continuously shaken in 25 ml. aerated solution. The solutions were renewed in fixed time intervals, *viz.* 0—5, 5—15, 15—30, 30—60, 60—120 minutes and so on, by means of which the interference between anions could be followed during both initial and continuous salt absorption.

Unlike the widespread occurrence of well developed antagonistic effects

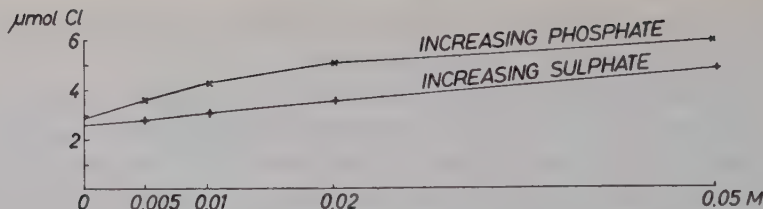


Figure 1. The relation between absorption of chloride (μmol . per 1 g. fresh weight) and rising concentration of added phosphate or sulphate (M). Basic cation potassium. Chloride was present in 0.005 M in all solutions. Potato tissue.

between almost all cations belonging to the alkali and earth alkali series the observed antagonistic effects between anions are less prominent. Noteworthy effects were observed only regarding $\text{Cl}^- \rightleftharpoons \text{NO}_3^-$.

Besides of antagonistic effects also synergistic effects may be observed, viz. stimulation of the absorption of one ion by the presence of another one.

Synergistic effects were observed at low concentrations (< 0.005 M) between most of the tested anions and at higher concentrations also with PO_4 contra Cl and SO_4 contra Cl (Figure 1). The presence of nitrate improved the initial absorption of phosphate in the combinations 0.005 M $\text{PO}_4 + 0.005$ —0.010 M NO_3 (Figure 3).

Synergistic effects were earlier observed also regarding cations. In this case, too, the phenomenon is restricted to low salt concentrations. This stim-

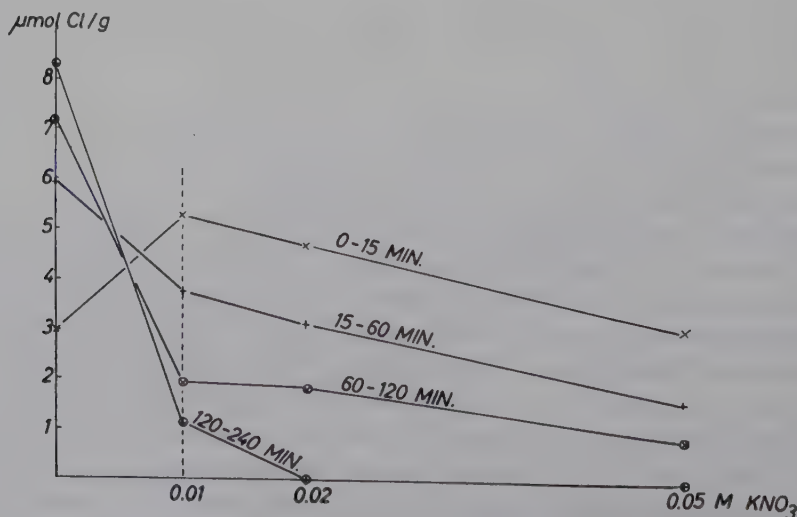
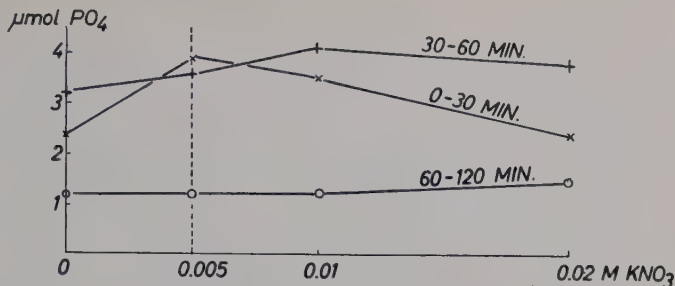


Figure 2. Influence of rising concentrations of potassium nitrate on the absorption of chloride (μmol . per 1 g. fresh weight) from 0.005 M KCl. Potato tissue.

Figure 3. Influence of rising concentrations of potassium nitrate on the absorption of phosphate (μmol . per 1 g. fresh weight) from 0.005 M KH_2PO_4 . Potato tissue.

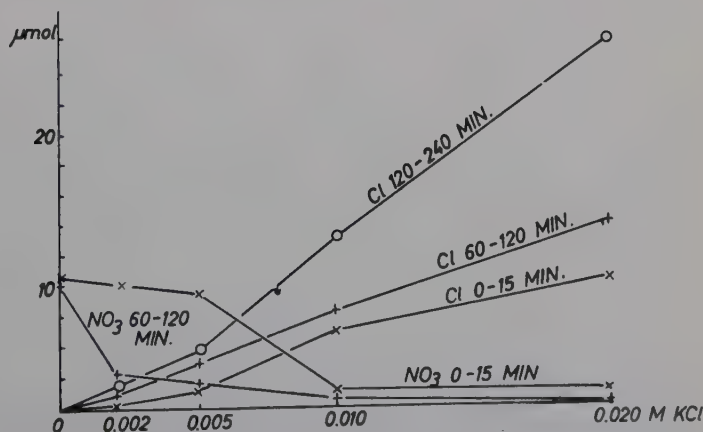


ulating effect may tentatively be explained from an interference of metabolic processes. Potassium and calcium are both needed for normal activity of the protoplasm. If the minimum quantities needed for normal metabolic work are sinking below the mark each rise of concentration will yield a positive effect. Of the tested anions NO_3 , PO_4 and SO_4 belong to nutrients which serve as fuel for indispensable biochemical processes.

Antagonistic effects of one anion upon the absorption of another anion are most clearly observed in the region above the "critical values" *viz.* at concentrations above 0.005 M of the dominating ion, but their appearance is also influenced by the time, *viz.* the periods of initial absorption and of continued accumulation may show a different response.

Clear mutual antagonistic effects were observed only regarding Cl and NO_3 (Figures 2, 4). A certain antagonism was also observed between Cl and PO_4 (Figure 5). Low concentrations of nitrate may again act stimulating on the initial absorption of chloride (see above). The antagonism NO_3 contra Cl develops during the *period of continued accumulation* (Figures 2, 4) and may lead to suppression of the active uptake of Cl. High concentrations of

Figure 4. Influence of rising concentrations of chloride on the absorption of chloride and nitrate. Nitrate was present in 0.005 M in all solutions. Wheat roots.



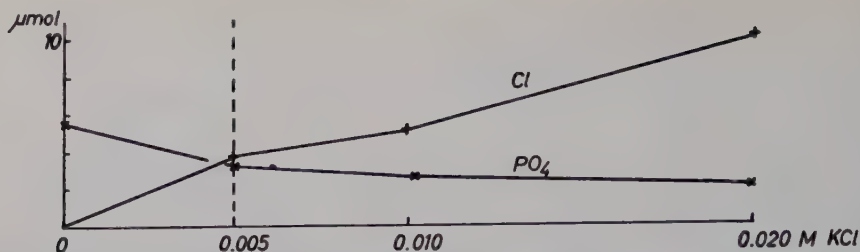


Figure 5. Influence of rising concentrations of potassium chloride on the absorption of chloride and phosphate. Phosphate was present in 0.005 M in all solutions. Potato tissue.

chloride have a similar effect on the active uptake of NO_3 . A depression of the active uptake of phosphate was observed under the influence of rising concentrations of chloride (Figure 5), whereas no such effect is noticed on the absorption of chloride in the presence of phosphate or sulphate (Figure 1).

Discussion

If ion antagonism is regarded from the view-point of ion exchange it must be concluded that two ions which are exhibiting mutual antagonism are *competing for the same carrier*. This conclusion does of course not exclude the existence of a certain degree of "selectivity", similar to that observed in ion exchange resins.

From the same view-point it may be concluded that weak or indistinct antagonism between actually adsorbed anions points to the occurrence of "specific" binding of anions besides of the unspecific, exchangeable adsorption. It may be hypothetically assumed that the metabolically active anions, *e.g.* NO_3^- , H_2PO_4^- , SO_4^{2-} , are caught by sites which are locally related to special biochemical mechanisms.

If ion antagonism is caused by metabolic competition, such an effect would probably appear preferably during the active continued salt accumulation. The strong increase of the antagonism between NO_3 and Cl during the period of continued accumulation (Figures 2 and 3) points to an influence of metabolic competition prior to exchange competition between anions. Further experimental work is needed for elucidation of these questions.

Summary

The mutual effects of anions in the process of salt absorption may be synergistic or antagonistic. Synergistic effects commonly appear at low con-

centrations and may be caused by metabolic interferences. Phosphate and sulphate act positively on the absorption of chloride even at high concentrations. Clear antagonistic effects were observed only at high concentrations of nitrate contra chloride.

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Investigations on the Mechanism of Absorption and Accumulation of Salts, V.

Retention and Eluation of Absorbed Salts

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I. Introduction

As shown in previous communications salt uptake in plants proceeds in two steps, initial absorption and continued accumulation. The initial absorption may be characterized as adsorption (on the basis of ion exchange) to carriers R^- and R^+ in the cytoplasm. Diffusion is also at work in the initial absorption, but is here a factor of secondary importance. Initial absorption runs with a low temperature coefficient and it is by definition reversible, *viz.* once absorbed ions may be exchanged with other ions or slowly washed out by distilled water.

Because the cytoplasm separates two aqueous phases, the medium and the sap space, a bilateral steady state is developed between the concentration of salt ions in the two aqueous phases and in the cytoplasm, a steady state which at complete equilibrium under ideal conditions would result in the same salt concentration in the sap as in the medium and the salt concentration in the cytoplasm amounting to a value which exceeds that in the aqueous phases by a factor of adsorption.

In roots or slices of storage tissue the process of initial adsorption is nearly completed in about 15 minutes. To what extent the adsorbed salts are going over into the cell sap in this brief period will depend upon the power of retention in the cytoplasm. Experiments with salt solutions, to which cyanide

was added, showed that in 15 minutes only little salt is transported by diffusion through a 0.5 mm. thick tissue.

The continued accumulation primarily implies the active transference of the initially adsorbed salt ions into the vacuoles. This process, which runs with a high "biochemical" temperature coefficient, gathers full speed in about 30 minutes from the start. Owing to the active accumulation no true adsorption equilibrium is attained in aerobically respiring cells, only a *dynamic steady state*, the up-hill side of which is composed by initial adsorption + active accumulation. The down-hill side of the dynamic state involves "passive leakage", the main driving force of which is the diffusion gradient, *viz.* the rising level of accumulation in the sap spaces as compared with a low concentration in the medium.

In the previous communications of this series the up-hill side of the dynamic steady state of salt storage in plant cells has been elucidated. The present communication deals with the down-hill side, *viz.* the rate of out-flow of once absorbed anions and the degree of retention of salts in aerobically respiring tissues.

II. Experimental Results

The experimental technique was described in previous communications (Lundegårdh 1958 a—c). Desalted roots of wheat or Triticale, and washed slices of potato tissue, were "charged" with salts (aerated and stirred 0.005 *M* solutions of KCl, KH_2PO_4 or KNO_3) in periods of 30 minutes or 24 hours, respectively. The accumulated quantities were determined from the losses in the medium and from analysis of the tissues. The charged tissues were vigorously washed in distilled water for 20 seconds, in order to remove adhering salt, and then transferred to an aerated and stirred volume of distilled water or solutions of α -dinitrophenol (DNP) or potassium cyanide (mostly 2—4 g. material in 50 ml. liquid). The medium was changed in appropriate intervals (see the figures 1—4) and analysed for exuded anions (Cl, PO_4 , or NO_3). After finished exudation (4 or 24 hours) the material was extracted in boiling water and the retained quantities determined. Conventional methods, described in the previous communications, were used for the chemical determinations. Care was taken to remove cyanide before determining Cl and chloride before determining NO_3 (Burstrom 1942). All experiments were carried out at 18°C.

If potato tissue is charged with *chloride* in a period of 30 minutes (*short charge*) about $\frac{1}{3}$ of the absorbed quantity (2.0 $\mu\text{mol./g.}$) is again exuded, if the material is washed in aerated distilled water during 30 minutes (Figure 1). It may be assumed that the quantities of chloride taken up in 30 minutes are chiefly initially absorbed, *viz.* that they largely remain in the cytoplasm. It must be postulated, however, that the outflow of salt anions is bilateral, *viz.* simultaneously to medium and sap space. This makes a total outflow of *in maximum* about $\frac{2}{3}$ of the initially absorbed salt in 30 minutes. Actually the

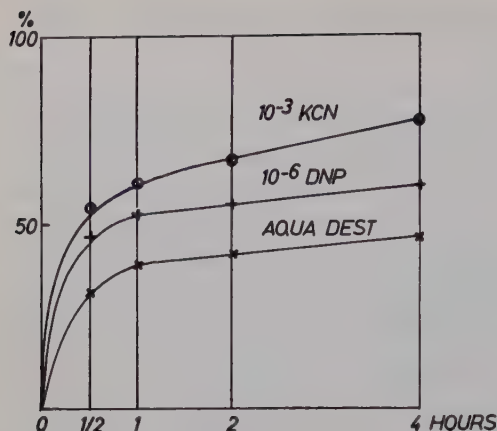


Figure 1. Elution of chloride from potato tissue in media consisting of distilled water or solutions of KCN and α -dinitrophenol. Short charge. Elution in % of total chloride (2.0 $\mu\text{mol./g.}$).

real outflow in the sap space during the first period of 30 minutes may be lower than that through the outer plasma membrane, because the tonoplast is less permeable, but the slowly starting active accumulation, too, acts in positive direction, if the medium is distilled water. In cyanide or DNP the active component is eliminated. In 15 minutes, or the postulated duration of the period of initial absorption, the outflow into the sap space will amount to in maximum about 25 % of the absorbed quantity. The calculated retention in the cytoplasm would then be only about 50 % in 15 minutes, and 33 % in 30 minutes.

During *continued washing* in non-poisonous media certain quantities of the initially absorbed salts are of course actively transferred to the vacuoles.

If the tissues are submitted to a *long charge* (24 hours) in 0.005 M KCl the internal concentration rises to 10 $\mu\text{mol. Cl/1 g.}$ Of this quantity about 8.5 % flows out again in 30 minutes, if the tissue is transferred to distilled water. As shown in Table 1 the removed quantities are thus about the same as those following upon a "short charge" (30 min.), notwithstanding the fact that the accumulated quantities of Cl are 5 times higher. This result shows that the level of adsorption in the cytoplasm remains about the same during long and short charge, an interesting fact because it shows that the rising concentration of the cell sap does not retroact on the adsorption in the cytoplasm. The active mechanism pushing anions from the cytoplasm into the sap obviously counteracts a readsorption of the once accumulated quantities. Only an increasing concentration in the medium is able to raise the level of initial absorption to higher levels (examples are given in Lundegårdh 1958 a—c).

The experiments quoted in Figures 1 and 2 show that the outflow of chloride from the vacuoles to the cytoplasm is a much slower process than

Table 1. *Potato tissue. Differences in the exudation of chloride following upon an initial charge (30 min.) or a long lasting charge (24 h.) with KCl. Values in $\mu\text{mol. per 1 g. fr. wt.}$*

Charge and Exudation period	Medium		
	Aqua	$10^{-6} M$ DNP	$10^{-3} M$ KCN
A. Short charge: 2.5 $\mu\text{mol. Cl/1 g. in 30 minutes}$			
Exudation in $\frac{1}{2}$ h.	0.83	1.17	1.37
Exudation in 4 h.	1.00	1.27	1.97
B. Long charge: 10.0 $\mu\text{mol. Cl/1 g. in 24 hours}$			
Exudation in $\frac{1}{2}$ h.	0.85	1.70	2.00
Calculated initial exudation	0.78	1.37	1.57
Calculated continued exudation	0.07	0.33	0.43
Exudation in 4 h.	1.50	1.70	2.30
Calculated initial exudation	1.18	1.50	2.00
Calculated continued exudation	0.32	0.20	0.30

the outflow from the cytoplasm to the medium. The degree of retention of *accumulated* chloride is fairly high, a fact which is obviously caused by the continuously acting anion respiration. The active process throws outflowing anions back again into the sap space. As a consequence of that it may be expected that inhibition of the mechanism of accumulation will accelerate the outflow of anions. Experiments with $10^{-3} M$ KCN and $10^{-6} M$ DNP show that this is actually the case (Figures 1 and 2).

Previous experiments showed that cyanide inhibits both initial absorption and accumulation. It was assumed that the anion carriers are largely identical with that part of the cytoplasmic structure (mitochondria etc.) which is the site of the cytochrome system and that the adsorptive qualities are closely related to an undisturbed activity of this system. But the mitochondria etc. are not only adsorbing anions, they are also by means of the intact cytochrome system transferring the salts to the sap space. If the active component, which returns escaping anions to the sap, is eliminated more salts are as a consequence flowing out from the vacuoles. But the rapid transition through the cytoplasm is gone with the elimination of the carrier activity and the out-flow of anions thus *limited to diffusion*, which is a considerably slower process than transport along adsorption tracks. This is probably the reason why the accumulated chloride is not too rapidly flowing out again if cyanide or DNP are applied. Earlier experiments have shown that the acceleration of the outflow in cyanide may be comparatively limited. The dimensions of the cytoplasm and the permeability of the tonoplast are here probably controlling factors.

DNP causes a partial disintegration of the structural qualities of the cytoplasm and increases the permeability of the tonoplast, thus still more accelerating the backflow from the vacuole through the inactivated cytoplasm. It was earlier shown that fluoride may yield similar results (Lundegårdh 1949,

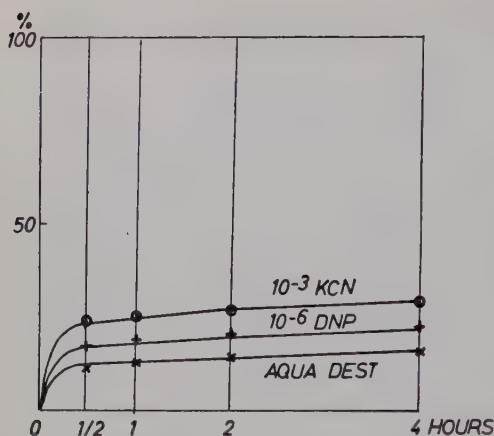


Figure 2.

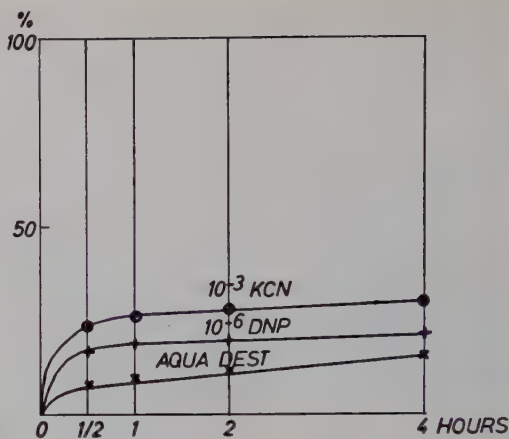


Figure 3.

Figure 2. Elution of chloride from roots of *Triticale* in a medium of distilled water or solutions of KCN or α -dinitrophenol. Long charge. Elution in % of total chloride ($5.9 \mu\text{mol./g.}$).

Figure 3. Elution of chloride from potato tissue. Long charge. Elution in % of total chloride ($10 \mu\text{mol./g.}$).

1950, 1952). The effect of DNP, the entrance of which is controlled by its dissociation (Stenlid 1949), remains lower than that of $10^{-3} M$ KCN as long as the concentration remains low (10^{-8} — $10^{-6} M$). $10^{-5} M$ DNP raises the exudation of chloride about twofold as compared with cyanide; similar effects were earlier obtained with fluoride (Lundegårdh 1949, 1950, 1952).

The outflow of absorbed chloride proceeds in much the same way in both potato tissue and wheat roots. It ought to be mentioned here that roots are exuding salts two ways: through the epidermis and through the vascular epithelium (Lundegårdh 1950).

The reason why accumulated chloride is retained to a considerable extent in the cells is not quite clear. Chloride is sometimes fixed in a way which resists any attempt to remove about 50 % of the absorbed quantities by boiling in aqua, hydrogen peroxide or acetic acid. The slow outflow of accumulated chloride may thus partly depend upon this tendency to fixation.

As to *phosphate* only a few percent of the absorbed quantities are assimilated in organic compounds. The accumulated inorganic phosphate is easily removed by boiling water. The accumulated quantities are, however, as in the case of chloride, largely retained in the tissues and only partially washed out in 48 hours. Washed roots of wheat or *Triticale* contain about $7.5 \mu\text{mol. PO}_4$ per 1 g. fr. wt.

In 30 minutes (short charge) about $2.0 \mu\text{mol./1 g. PO}_4$ are absorbed from

Table 2. *Roots of Triticale. Differences in the exudation of phosphate following upon an initial charge (30 min.) on a long lasting charge (24 h.) with KH_2PO_4 , pH 5.0. Values in $\mu\text{mol. per 1 g. fr. wt.}$*

Charge and Exudation period	Medium		
	Aqua	$10^{-6} M \text{ DNP}$	$10^{-3} M \text{ KCl}$
A. Short charge: 9.5 $\mu\text{mol./1 g.}$ in 30 minutes. Extant charge 7.5, new charge 2.0 $\mu\text{mol./1 g.}$			
Exudation in $\frac{1}{2}$ h.	1.52	1.29	1.31
Exudation in 4 h.	1.76	1.75	1.80
B. Long charge: 13.6 $\mu\text{mol./1 g.}$ in 24 h. Extant charge 7.5, new charge 6.1 $\mu\text{mol./1 g.}$			
Exudation in $\frac{1}{2}$ h.	2.06	4.90	2.58
Calculated initial exudation	1.50	1.29	1.31
Calculated continued exudation	0.56	3.61	1.27
Exudation in 4 h.	2.72	6.80	3.40
Calculated initial exudation	1.76	1.75	1.80
Calculated continued exudation	0.96	5.05	1.60

0.005 $M \text{ KH}_2\text{PO}_4$ (pH 5). This quantity is almost completely washed out in 4 hours (Table 2). The total absorbed quantity is in this case $7.5 + 2.0 = 9.5 \mu\text{mol./1 g.}$ Only the "extra" PO_4 is thus going out again.

In 24 hours (long charge) the absorbed quantity amounts to $6.1 \mu\text{mol./1 g.}$ and the total accumulation to $6.1 + 7.5 = 13.6 \mu\text{mol./1 g.}$ In 4 hours only about 45 % of this "extra" quantity is washed out in aqua. Because in *maximum* about 1.75 $\mu\text{mol.}$ of this quantity refers to exudation of initially adsorbed quantities 0.96 $\mu\text{mol.}$ probably comes from the vacuoles. As compared with chloride phosphate thus penetrates the tonoplast a little more easily. The fact that now a basic quantity of $7.50 \pm 3.38 = 10.88 \mu\text{mol./1 g.}$ resists outwashing points to a fairly strong retention of accumulated phosphate. The diffusion along the gradient cell sap \rightarrow medium is counteracted by the accumulating activity of the anion respiration, but besides of that the cytoplasm and the tonoplast are raising a considerable resistance against the outflow. This conclusion is supported by the comparatively low effect of cyanide (Table 2). In 4 hours the outflow here only rises from 2.72 to 3.40, a quantity referring to accumulated, not to initially absorbed phosphate.

Cyanide is comparatively more efficient in facilitating the outflow of chloride than that of phosphate. This fact possibly points to the existence of different carriers for these two anions (*cf.* Lundegårdh 1959 a), but attention may also be called to the fact that, besides of the anion respiration, special chemical mechanisms are at work in the phosphate metabolism. These are not inhibited by cyanide. It was shown, however, (Lundegårdh 1958 b), that these special mechanisms are only little interfering with the general mechanism of accumulation.

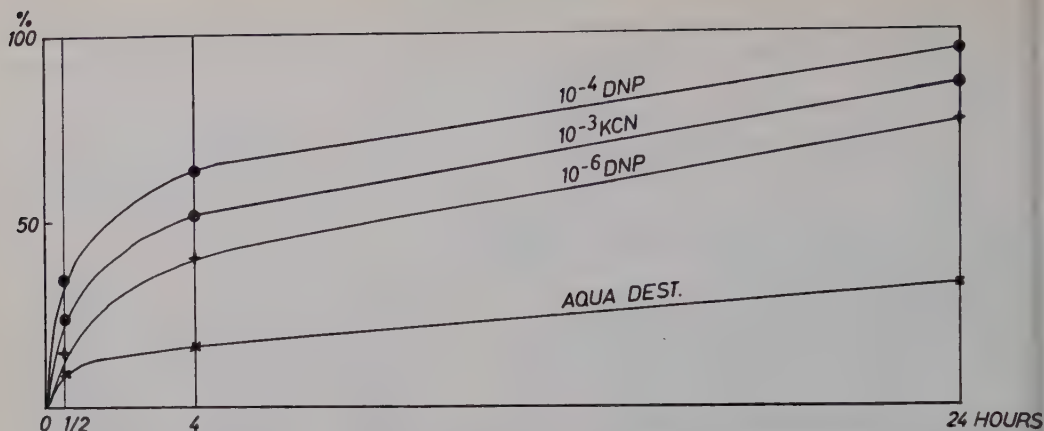


Figure 4. Eluation of nitrate from roots of *Triticale* in a medium of distilled water or solutions of KCN and α -dinitrophenol. Long charge. Eluation in % of total nitrate (63–83 $\mu\text{mol./g.}$).

A number of processes, in which inorganic phosphate is involved, are on the other hand strongly affected by dinitrophenol. This agent is looked upon as an universal inhibitor of the formation of high energy phosphate. $10^{-6} M$ DNP does not increase the outflow of initially absorbed phosphate (short charge), whereas the outflow of accumulated quantities (long charge) more than twofold exceeds the exudation in aqua destillata (Table 2). Nevertheless DNP does not increase the outflow significantly above the level of "extra" phosphate in 4 hours.

Compared with chloride and phosphate nitrate is more intensively absorbed. From a $0.005 M$ solution of KNO_3 up to $84 \mu\text{mol./1 g.}$ were accumulated during 24 hours in roots of wheat and *Triticale* (Table 3; fig. 4.), corresponding to an osmotic pressure of about 4 atmospheres.

Of the accumulated nitrate (long charge) about 14 % is given off to aqua destillata in 4 hours. This figure is not very much lower than the *relative* exudation of chloride and phosphate (15 or 20 % respectively) following upon a long charge, but the *absolute* quantities are much higher, viz. $14.0 \mu\text{mol./1 g. NO}_3$ against 2.72 PO_4 and 1.50 Cl . These figures point to a direct relation between the height of accumulation and the intensity of the passive outflow, as may be expected from a process which is controlled largely by diffusion. It ought to be mentioned here that the active accumulation within a large region of concentrations rises approximately in line with the salt concentration. The steady state between outflow from the vacuoles and the active return of the salts to the sap spaces (see above) does consequently not appreciably interfere with the simple rules of diffusion which

Table 3. *Roots of Triticale. Long charge: 83.0 μ mol. nitrate/1 g. fr. wt. in 24 h. Exudation and retention determined by analysis of the medium and the root material* Values in μ mol. per 1 g.

Fraction of nitrate	Medium			
	Aqua	10^{-6} M DNP	10^{-4} M DNP	10^{-3} M KCl
Exudation in $\frac{1}{2}$ h.	9.2	11.2	22.3	22.6
Exudation in 4 h.	14.0	29.7	64.7	43.5
Exudation in 24 h.	25.7	45.4	79.0	57.5
Retention in 24 h.	55.0	35.5	17.4	28.0
Retention plus Exudation	80.7	80.9	96.4	85.5
Deviation from determined charge	- 2.8 %	- 2.4 %	+ 16 %	+ 3 %

are reflected in the passive outflow. Individual differences are of course observed, depending upon "permeability", *viz.* the presence of various controlling factors in the path of diffusion.

The outflow of nitrate is considerably more facilitated by the presence of 10^{-3} M KCN than was phosphate (Table 3; Figure 4). In 24 hours 31 % NO_3 is lost to aqua dest. and 70 % to 10^{-3} M KCN. 10^{-6} M DNP acts here, contrary to the phosphate series, weaker than 10^{-3} M KCN, whereas in 10^{-4} M DNP the total quantity of nitrate nitrogen is lost in 24 hours. Table 3 shows the good agreement between determinations of nitrate in solution and nitrate in the tissues. The "super"-exudation in 10^{-4} M DNP points to some organic nitrogen exuded as nitrate.

III. Discussion

The experimental results emphasize the mobility of the initially absorbed salts, *viz.* salt transport in the cytoplasm. Of salts absorbed in the cytoplasm about $\frac{1}{3}$ is washed out again in 30 minutes. These quantities are eluted from the bulk of the tissue. The comparatively high mobility of salts in root tissues was shown in previous investigations regarding sap movement (Lundegårdh 1950).

Experiments on the absorption of chloride and nitrate at inhibited active accumulation (10^{-3} to $2 \cdot 10^{-3}$ M KCN added to the salt; Lundegårdh 1958 a and c) showed that certain quantities of salts are entering the tissues by means of *diffusion*. The velocity of diffusion varied considerably in the single experiments and values from 5 to 50 % of the quantities absorbed in the absence of cyanide were recorded. It was concluded that diffusion is always participating in uptake and distribution of salts, but that this process as a rule proceeds at a considerably lower speed than initial absorption in an intact cytoplasm. However, the fact that the velocity of elution is high, if the

co-operation of carriers is reduced to a minimum, *e.g.* under the influence of cyanide, points to diffusion as the dominating process of outflow. Acceleration of the eluation simultaneously with the inactivation of carriers may be explained simply from a rise in the diffusion gradient following upon the release of the initially absorbed salts.

Kling (1958) has recently called attention to the existence of structural pathways for transport of anions. These tracks are according to Kling running from the surface of the cells to the tonoplast and may be identified with fibrous mitochondria. These observations are in accord with results established in this laboratory (Lundegårdh 1958 c) regarding the identity of those structures, which carry cytochromes, with those particles which constitute the visible structure of the cytoplasm. Kling found interesting relations between these anion carrying structures and patches of "Kalllose" on the cell walls. These patches are possibly serving as transport vehicles from one cell to another.

It was previously shown that the process of continued accumulation of salts in the vacuoles starts from those quantities of anions, which are initially absorbed in the cytoplasm. *Inactivation of the carriers thus abolishes accumulation, even if the cytochrome system remains partially intact, e.g.* under the influence of DNP or fluoride. These agents are accelerating the outflow from the cytoplasm but leave the activity of the cytochrome oxidase and cytochrome c more or less intact. An irregular response of cytochrome b reflects the inhibition of phosphorylation and the subsequent inactivation of the carriers. The conclusion, drawn by a number of author's, that phosphorylation in itself might serve as an universal mechanism of salt accumulation, is not supported by observed facts, as shown in previous discussions of this question (Lundegårdh 1952, 1954, 1958 a—c).

The experimental results yielded the conclusion that the outflow of salts from the vacuoles is considerably slowed down as compared with the eluation from the protoplasm. Regarding intact tissues, which are washed in distilled water, the explanation of these observations encounters no difficulties. The process of active accumulation continues and a steady state is slowly attained, in which active accumulation compensates for losses by diffusion. An example of this steady state is given by the experiments with phosphate: Only the "extra" charge is easily eluated, whereas a certain "stock" of phosphate successfully resists outwashing during 24 hours. It is a well known fact, however, that the steady state at prolonged washing gives way to the outgoing salt stream, finally yielding a more or less "desalted" tissue. The velocity of the outwashing is fairly individual, some tissues, *e.g.* in roots, are comparatively rapidly losing salts, primarily through the central sap stream. Some salt anions, *e.g.* nitrate, are lost more rapidly than

others, *e.g.* phosphate, and so on. The principal thing is here that the process of active accumulation always implies the maintenance of an ingoing stream of anions which, if the medium contains a minimum of salts, detains a dynamic state of super-pressure in the vacuoles.

It was previously (Lundegårdh 1959 a) shown that anions may be selectively adsorbed. The elution of charged tissues, too, reveals individual differences between the three anions Cl , H_2PO_4 , and NO_3 , of which NO_3 is more willingly eluted than the two others. Because the differences are observed both with and without inhibitors the permeability of the tonoplast is probably a regulating factor. A special biochemical effect is probably involved in the somewhat deviating response of phosphate to DNP and KCN (Table 2). The *initial absorption* remains practically unchanged, whereas primarily DNP strongly affects the outflow from the vacuoles. DNP accelerates the reaction $\text{ATP} \rightarrow \text{ADP} + \text{PO}_4$, which is again retarded by an increase in the concentration of inorganic phosphate. If inhibited formation of ATP contributes to the inactivation of the anion carriers (Lundegårdh 1958 a) a high charge of phosphate would accordingly counteract the destructive effects of DNP and KCN. The inhibition may, however, be sufficient for a retardation of the process of accumulation, as a consequence of which some phosphate is flowing out from the salt stores in the vacuoles.

Summary

Experiments on retention and elution of absorbed chloride, phosphate or nitrate shows that about 30 % of initially absorbed salt ("short charge") are eluted in less than 4 hours. The process is akin to the elution from an adsorbent and is largely controlled by diffusion. Virtually accumulated salts ("long charge") are only slowly eluted by distilled water owing to the continuously proceeding process of accumulation (anion respiration).

Cyanide and α -dinitrophenol are both accelerating the elution, because the adsorptive power of the cytoplasm (=the anion carriers) is abolished. Certain specific differences between the anions are observed.

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Light Adaptation in Marine Phytoplankton Populations and Its Interrelation with Temperature

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Introduction

Until recently very few investigations were designed to study the influence of varying light intensity on the photosynthetic rate in natural phytoplankton populations. The investigations by Steemann Nielsen 1937 represent an early marine contribution.

During the last few years, however, interest in the problems has increased considerably. Contributions have been published both by limnologists — Talling 1957, Verduin 1957, Edmondson 1957 — and by marine workers — Ryther 1956, Steemann Nielsen and Aabye Jensen 1957. Finally the results from the many investigations dealing with big scale cultivation of plankton algae must be mentioned — *cf.* Burlew 1953, Tamiya 1957. In many respects such investigations are of considerable importance for understanding the processes going on in Nature.

One special problem concerning the light influence in the photosynthesis of natural phytoplankton populations has been much neglected, however, namely the problem of light adaptation. Only little is mentioned in the literature. Gessner 1949 working in a Bavarian lake showed that the assimilation number, *i.e.*, the quotient of assimilated CO_2 per hour at optimum light, approached the average of 10 at the surface during most of the year. In the hypolimnion, however, the assimilation number generally was definitely lower.

Even investigations on light adaptation under laboratory conditions using cultures of single plankton species are very scarce. The most notable contributions are those of Sargent 1940 and Winokur 1948.

In the mechanism of photosynthesis both photochemical processes and enzymatic processes take part. At low light intensities, the photochemical processes determine the rate of photosynthesis. The initial slope of a curve for phytoplankton showing the rate of photosynthesis as a function of light intensity is thus proportional to the light absorption by the active pigments; *cf.* Gabrielsen 1948. It is also dependent on, but not strictly proportional to — *cf.* p. 364 — the concentration of active pigments, especially chlorophyll *a*. At high light intensities no dependency is found. Instead the concentrations of the enzymes taking part in photosynthesis determine the rate of the whole process. If we disregard differences in the initial slopes of the curves, light adaptation may be defined as the adjustment of the rates of the enzymatic processes taking part in photosynthesis. This adjustment is a combined effect of enzyme concentration and temperature. But let us initially disregard the influence of temperature.

Special euphotic and oligophotic species of plankton algae have only been found in the tropical-subtropical parts of the ocean, as by Karsten 1907. As will be shown in this article, the populations of non-tropical plankton algae at the different depths, although identical with respect to the occurrence of species, nevertheless physiologically may behave quite differently. The different species must be able to adapt their behaviour to light according to the light conditions found at the different depths.

The hydrographical conditions of a certain area determine the degree of differentiation in the light adaptation between surface plankton and sub-surface plankton. A considerable degree of vertical stabilization of the water masses in the photosynthetic zone, preventing any extensive vertical mixing, is necessary in order to create a typical differentiation in the light adaptation of the algae within the photosynthetic zone. In the shallow photic zone in lakes the extent of the vertical mixing of the water masses during the day is often too considerable. In the sea, in several shallow areas or in areas with strong tidal currents, the conditions are similar. On the other hand, in many other marine areas the hydrographical conditions within the photic zone are such that the water masses found at the different depths do not mix or mix only slowly. Under such circumstances, through light adaptation, plankton populations with different behaviour are produced in the different layers.

Besides the light adaptation due to the vertical distributions of the algae, a similar adaptation is attributable to the different seasons of the year or to the different latitudes. In such cases, however, it is often difficult to decide whether the different behaviour of the plankton observed is due to light

adaptation, to differences in temperature or to the differences in the composition of species. The difference in temperature was thus suggested by Talling 1957 to be the main factor responsible for the different types of curves showing the rate of photosynthesis as a function of light in the freshwater diatom *Asterionella formosa* collected at different seasons in some English lakes.

1. *Photosynthetic characteristics of plankton populations*

Due to the complexity of photosynthesis it is rather unlikely that a mathematical equation can be found to describe in an unequivocal way the photosynthesis as a function of light intensity. The empirical equation proposed by Smith 1936, however, to some extent may be considered useful to relate photosynthetic rate (P) and light intensity (I):

$$K \cdot I = P / (P_{\max}^2 - P^2)^{1/2}$$

where K is a constant that locates the curve upon the rate of light intensity and P_{\max} is the rate of light saturated photosynthesis, *i.e.* the asymptotic maximum rate of photosynthesis. The equation has been used to describe the shapes of curves found in plankton algae; *cf.* for example Winokur 1948, and Talling 1957.

Talling 1957 introduced a quantity I_K to define the onset of light saturation of photosynthesis. It has been found useful as a photosynthetic characteristic of plankton populations, since it may be used even when the shape of the curve does not strictly conform to the above equation. I_K denotes the reciprocal K^{-1} if in the Smith equation P_{\max} is put at 1 and $p = P/P_{\max}$ is introduced. I_K is equivalent to the light intensity at which in a photosynthesis-light intensity curve the extrapolation of the initial linear region and the light-saturated region intersect. It is thus easy to determine I_K .

Photosynthesis intensity curves of optically thin suspensions of plankton algae may be presented in the following three ways:

a) as relative-curves where the rate of light-saturated photosynthesis is put at 100. The curves presented in Figures 1, 4, 6, 7, 8 represent this type.

b) curves presenting absolute rates of photosynthesis per definite amounts of pigments. The curves presented in Figures 2, 5, 9, 10 belong to this type. If no measurements of the pigments have been made, a value of 0.36 mg. C assimilated per mg. chlorophyll per hour at a light intensity of 1000 lux may be employed as a useful approximation; *cf.* Steemann Nielsen and Aabye Jensen 1957.

c) curves giving the rate of photosynthesis per weight unit of organic matter found in the algae. Working with cultures of single species such as done by Winokur 1948 it is possible to make such curves adequately. Working with natural phytoplankton populations severe difficulties turn up, however. It is ordinarily of little value to determine by chemical means the organic particulate matter present; the main part of it may be found outside of the photoautotrophic algae. In practice two indirect methods are possible:

1) After enumerating the plankton algae and measuring their cell volumes the amount of organic matter is computed from the total cell volume by the use of a convection factor; 2) The organic matter is computed from the concentration of chlorophyll found using arbitrary convection factors valid for the different water masses.

In method 1) inaccuracies may be due to the enumeration, to the measurements of cell volumes, and to the choice of the convection factor between cell volume and organic matter. In method 2) the main difficulty is the choice of the convection factor between chlorophyll and organic matter. The present authors are inclined to use the second method although they are aware of its limitation.

In describing a photosynthesis light intensity curve completely, a fourth quantity is necessary besides P_{\max} , I_K , and the gradient of the curve at low light intensities. This quantity is the light intensity at which light inhibition sets in; cf. Fig. 1, Fig. 6, and Fig. 8. It will not be especially discussed in the present article. Light inhibition starts ordinarily at relatively low light intensities in shade plankton. As will be shown below, the photochemical processes of photosynthesis are very effective here due to a high concentration of pigment, whereas the rates of the chemical processes are low due to relatively low concentrations of the photosynthetic enzymes. Conditions for photooxidation thus exist.

2. "Sun" and "shade" plankton

In terrestrial plants it was found at an early stage, — starting with the work of Lubimenko — that leaves of some specimens of a plant species photosynthetically may act as "sun" leaves whereas other specimens act as "shade" leaves (for references see: Rabinowitch 1945, p. 422). The two types of leaves may even be found simultaneously on the same specimen of a tree. Per leaf surface or per weight, "sun" leaves are characterized by having both a high rate of photosynthesis at light saturation and a high rate of respiration. "Shade" leaves, on the other hand, have both a low rate of photosynthesis at light saturation and a low rate of respiration. The slopes of the photosynthesis light intensity curves in the two types of leaves are generally identical at low light intensities. The compensation point — i.e., the light intensity at which photosynthesis compensates respiration — is thus low for a "shade" leaf but relatively high for a "sun" leaf. "Shade" leaves are thus better adapted for the life at low light intensities. On the other hand, a "sun" leaf is best suited for the life at high light intensities.

Ruttner 1926 showed that the aquatic species *Elodea canadensis*, a phanerogamic plant, behaved quite differently physiologically according to the depth where it had grown. The specimens collected near the surface acted as "sun" plants, those taken from greater depths as "shade" plants. The same has been shown by several other workers concerning other sessile aquatic plants.

By growing *Chlorella* at different light intensities, Sargent 1940 and Winokur 1948 showed that plankton algae are able to produce light adapted cells in exactly the same way as trees are able to produce "sun" leaves and "shade" leaves. Certainly we are not bound to expect that all species of plankton algae are equally able to produce populations of typical "shade" cells and typical "sun" cells. In terrestrial plants the ability to produce "light" and "shade" leaves seems not to be equally developed in all species. Talling, 1957 in his study on the freshwater diatom *Asterionella formosa*, was unable to show such a light adaptation. His experimental procedure, however, was not suited — and not intended — for such an investigation. The behaviour in relation to light-saturation was dominated by the effect of temperature.

During the cruises of the Danish Fishery Research Ship "Dana" in the North Atlantic and in the Arctic 1956, 1957, and 1958, experiments were made with plank-

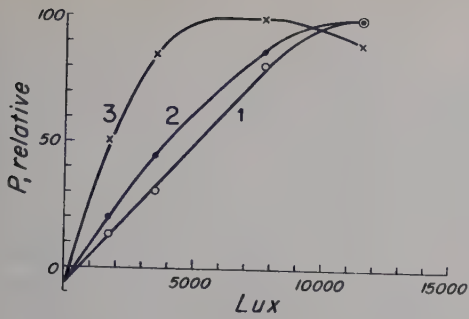


Figure 1.

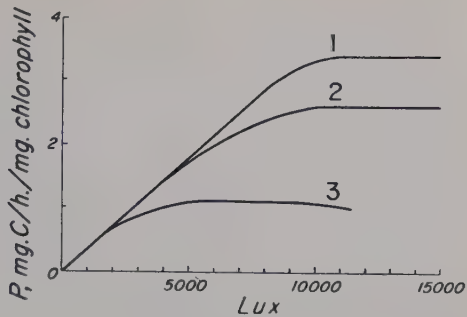


Figure 2.

Figure 1. Light intensity and relative rates of net photosynthesis in arctic summer plankton from three different depths, 1=surface, 2=27 m., 3=50 m. Dana Station 10531.

Figure 2. Light intensity and rate of gross photosynthesis per unit of chlorophyll. Dana Station 10531. 1, 2 and 3 as in Figure 1.

ton from all depth of the photic layer showing the rate of photosynthesis as a function of light intensity. These investigations were partly made to elucidate the light adaptation in natural populations of plankton algae in the sea. At the same time these investigations were used as a means for measuring the rate of respiration — by extrapolating the curve to light intensity 0. That part of the investigations is published elsewhere (Steemann Nielsen and Hansen 1959). The details of the technique are described in the other publications just mentioned. It is thus sufficient to present a short outline here. The carbon-14 technique — Steemann Nielsen 1952 — was used. The experimental bottles were placed on a rotating wheel in a water bath, the temperature of which was regulated to be the same as that found in the sea. The light was produced by a series of bulbs of incandescent light. The variation in light intensity was established by using neutral filters in front of the experimental bottles.

The shipboard installation so far employed permits the use of only five different light intensities, including the intensity 0 (=dark). If the main purpose is to determine the initial slope of the light intensity photosynthesis curve in order to extrapolate it for the measurement of respiration (Steemann Nielsen and Hansen 1959), preferably three of the single determinations have to be made in the initial straight part of the curve. If on the other hand the main purpose is to produce a general light intensity photosynthesis curve, such as presented in this paper, the single determinations must be distributed equally within the light range. By combining two single series of measurements made with the same plankton it is of course possible to use more different light intensities and to pay due regard to both instances; cf. Fig. 7.

Figure 1 present three curves showing the rate of photosynthesis as a function of light intensity in plankton from the Dana Station 10531 in the central part of the Davis Strait (between Greenland and Labrador) at latitude 64°N , in July, a) from the surface, b) from a depth of 27 m (10 % of the green light at the surface was found at that depth) c) from the lower part of

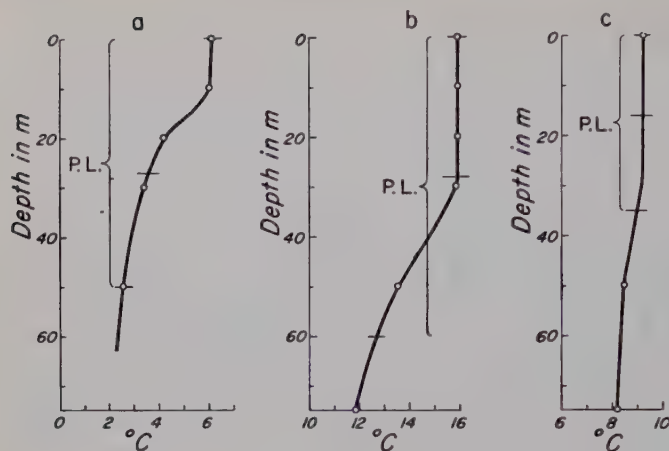


Figure 3. The vertical distribution of temperature at three Dana Station; a: 10531 — cf. Figure 1 —; b: 10986 — cf. Figure 8 —; c: 10460 — cf. Figure 4.

the photic zone at a depth of 50 m. (1 % of the green light at the surface). The curves are relative, light saturated photosynthesis in all cases being put at 100. The curves are corrected to true net production (Steemann Nielsen and Hansen 1959). The experiments with the plankton from the different depths were made at practically the same temperature, 4.5—6°C. Whereas light saturated photosynthesis is obtained at about 11,000 Lux in the surface plankton ($I_K=9,500$ Lux; cf. p. 355) and at 10,000 Lux in the plankton from 27 m. ($I_K=7,300$ Lux) it is obtained at about 5,500 Lux in the plankton from the greatest depth ($I_K=3,200$ Lux).

In Figure 2 the same three curves — corrected to true gross production — are presented in another way. The slopes of the curves at lower light intensities are now identical. The slope in dilute plankton suspensions is approximately proportional to the concentration of active pigments (cf. p. 355). The curves in Figure 2 present — approximately — photosynthesis as a function of light intensity per the same amount of active pigments (=1 mg. chlorophyll).

A relatively stable stratification of the water masses near the surface is found in that part of the Davis Strait in the middle of summer. Figure 3a presents the vertical distribution of temperature in the photosynthetic zone at Dana Station 10531. A typical vertical stratification of the water masses within the zone is found. Light adaptation of the phytoplankton is thus possible. Both according to Figure 1 and Figure 2, a significant difference is found in the photosynthetic behaviour of the surface plankton and the sub-surface plankton. Although the taxonomic composition of the plankton was not investigated, any idea of special "light" and "shade" species occurring in the Arctic may be disregarded. None of the many plankton surveys from

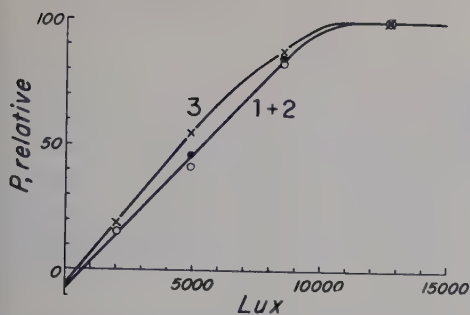


Figure 4.

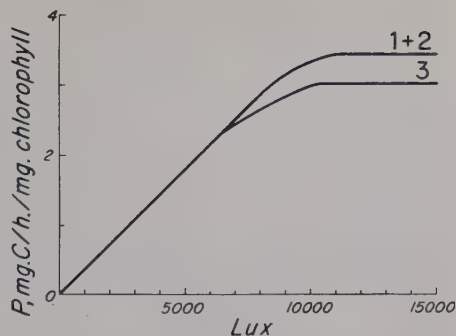


Figure 5.

Figure 4. Light intensity and relative rates of net photosynthesis in summer plankton from three different depths, 1=surface, 2=16 m., 3=35 m. Dana Station 10460.

Figure 5. Light intensity and rate of gross photosynthesis per unit of chlorophyll. Dana Station 10460. 1, 2 and 3 as in Figure 4.

the Arctic has shown any indication of the occurrence of a special "shade" flora. The occurrence of "sun" and "shade" adapted populations of the same species seems more likely.

Figure 4 presents two corrected curves of the rate of photosynthesis — net production — as a function of light intensity, a) a joint curve from the surface and from a depth of 16 m. (10 per cent. of the green light at the surface), b) from a depth of 35 m. where the green light was one per cent. of that at the surface. The plankton was collected at a Station over the submarine ridge between the Faroes and Iceland during July. In this case the two (three) curves were rather similar, I_K being 9,800 Lux at the surface and at the 10 % depth and 8,500 Lux at the 1 % depth. In Figure 5 the same curves are presented (corrected to gross production) computed per 1 mg. chlorophyll. Nearly no vertical stability of the water masses was found in the photic layer; cf. Figure 3 c. Such conditions impede the establishment of a pronounced light adaptation of the plankton algae.

In order to show that the curves presented above really represent the ordinary situation in the sea, some other series of experiments — out of a considerable collection of similar curves — are given in addition below. The curves are corrected illustrating net photosynthesis. The optimum rate is always put at 100. Figures 6 and 7 illustrate light intensity curves from two stations in the Davis Strait at the height of summer. Measurements were made with water from the surface and from the depths where 10 % and 1 %, respectively, of the green surface light was found. In principle the figures resemble figure 1 illustrating the light intensity photosynthesis curves from another station in the same area. Taking all three stations together the sur-

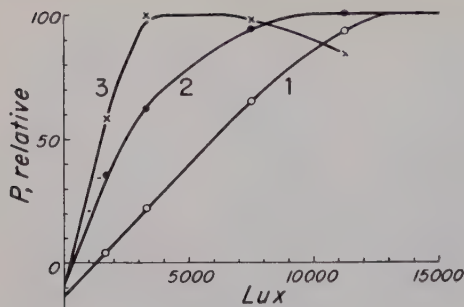


Figure 6.

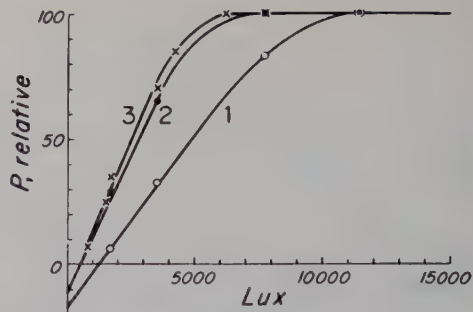


Figure 7.

Figure 6. Light intensity and relative rates of net photosynthesis in arctic summer plankton from three different depths, 1=surface, 2=25 m., 3=50 m. Dana Station 10561.

Figure 7. Light intensity and relative rates of net photosynthesis in arctic summer plankton from three different depths, 1=surface, 2=27 m., 3=52 m. Dana Station 10540.

face curves are very alike. The curves from 1 % depth are also rather alike. The curves from the 10 % depth are in all cases intermediate between the corresponding surface curve and the 1 % curve. However, whereas at Station 10531 the 10 % curve is very near to the surface curve at Station 10540 it is nearly identical with the 1 % curve.

Figure 8 illustrates light intensity photosynthesis curves from Station 10986 in the warm Atlantic Current at 51°N, in August. The surface curve (temperature 16°C) is rather like the corresponding curves from the arctic water in the Davis Strait made at about 5°C, even at latitude 69°N. The curve from the depth where 1 % of the green surface light was measured (temperature during the experiments 15°C) is even practically identical with the corresponding curves from the arctic water made at about 5°C. The curve from

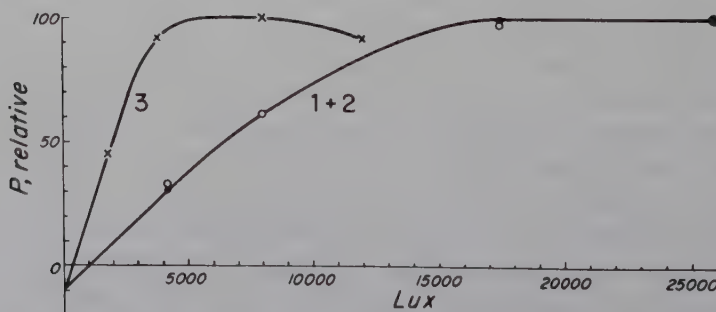
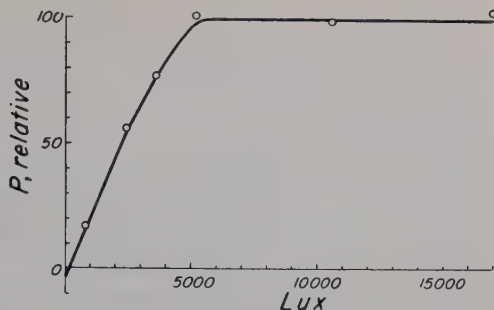


Figure 8. Light intensity and relative rates of net photosynthesis in temperate oceanic summer plankton from three different depths, 1=surface, 2=28 m., 3=60 m. Dana Station 10986.

Figure 9. *Light intensity and relative rates of net photosynthesis in temperate winter plankton from the surface. The Sound off Elsinore, 14. XII. 1958.*



the 10 % depth (Fig. 8) was completely identical with that from the surface. Figure 3 b showing the vertical distribution of the temperature at Station 10986 indicates that the 10 % depth is found in the vertically completely mixed surface layer which does not allow any establishment of differently adapted algae within the layer. The 1 % depth on the other hand was found below the vertically mixed surface layer, thus rendering the establishment of a shade plankton possible.

Figure 9 illustrates a light intensity photosynthesis curve from the surface at latitude 56°N — Sound off Elsinore — during midwinter ($t=6.3^{\circ}\text{C}$). It is similar to curves illustrating shade plankton during summer. As the measurements were made in the laboratory in Copenhagen, more different light intensities could be employed.

3. *A comparison between light intensity photosynthesis curves from different latitudes*

In Figure 10 a collection of some of the curves for gross production presented in section 2 are drawn together. The initial slopes of the curves are made identical, denoting that the photosynthetic rate is given per identical amount of active pigments. In addition two curves made with either tropical or temperature surface water are presented (according to Fig. 32 and Fig. 36, Steemann Nielsen and Aabye Jensen 1957).

Physiologically the set of curves shows that the efficiency of the chemical part processes in photosynthesis may vary extraordinarily from one population to another although the efficiency of the photochemical part processes is constant. The rate of a photochemical process is independent of temperature.

The rate of the chemical processes in photosynthesis is dependent a) on the concentration of the active enzymes, b) on the temperature. By increasing the temperature by 10°C the rate of the enzymatic process increases by

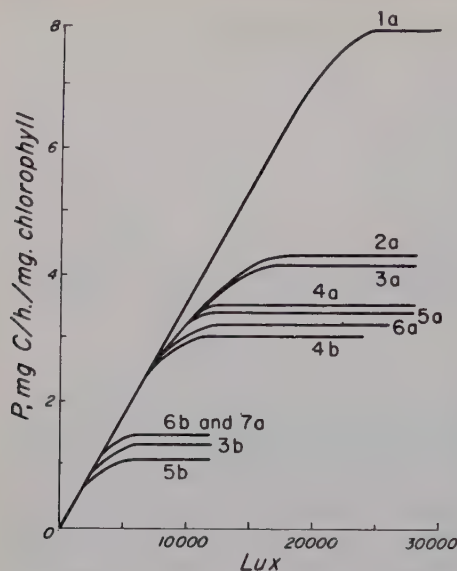


Figure 10. Light intensity and rate of gross photosynthesis per unit of chlorophyll, a = surface plankton, b = plankton from the depth at which one per cent of the green surface light was found. 1 = tropical plankton (Galathea Station 518), 2 = temperate summer plankton (Galathea Station 599 a), 3 = temperate summer plankton (Dana Station 10986), 4 = plankton from a northern area with very slight vertical stabilization (Dana Station 10460), 5 and 6 = arctic summer plankton (Dana Stations 10531 and 10540), 7 = temperate winter plankton (latitude 56°N).

a factor of between 2 and 3. In the following we will consider in a general way the limitations in rates due to the concentrations of the photosynthetic or respiratory enzymes, although strictly speaking only the concentrations of the respective rate-limiting enzymes are of importance.

The two shade curves 3b and 6b are nearly identical. However, the content of enzymes per unit of active pigments must be 2—3 times higher in 6b (shade plankton from 65°N) than in 3b (shade plankton from 51°N). The experimental temperature was 2°C in 6b and 15° in 3b. The ratio of pigments to organic matter is very likely practically the same in the two populations of plankton. It seems, at least, to be a very likely condition in two extreme shade populations. The ratio of the photosynthetic enzymes to organic matter in the algae may thus be estimated to be 2—3 times higher in the arctic plankton from 65°N than in temperate plankton from 51°N.

The curves 3a and 6a, illustrating photosynthesis in the surface plankton from respectively 51°N and 65°N, are also reasonably identical. As the difference in temperature in this case also is about 10°C, the content of the photosynthetic enzymes of the arctic plankton — computed per unit of active pigments — is at least twice as high as in the temperate plankton. As the difference in the ratio: active pigments to organic matter in the two plankton populations very likely is rather small, we may assume that the concentration of the photosynthetic enzymes in the algae cells of the arctic surface plankton is about twice of that in the temperate surface plankton.

Thus the photosynthesis light intensity curves of shade plankton and sur-

face plankton indicate that *in the arctic the influence of the low temperature on the rate of photosynthesis is counteracted by a higher concentration of enzymes.*

The differences in the behaviour at the same temperature between the shade populations on one hand and the surface populations on the other hand shows that the light adapted surface populations have relatively much higher contents of the enzymes per unit of the pigments as the shade plankton; *cf.* the curves 3 *a* and 3 *b*, 5 *a* and 5 *b*, and 6 *a* and 6 *b* in Figure 9.

In the shade populations the ratio: concentration of active enzyme to concentration of active pigments was both in the arctic and in the temperate water only about 25 per cent. of that of the surface plankton. We may simply use the saturation plateau of the different curves in Figure 10 for comparing the ratios in question.

The light intensities found at the surface increase going from high to low latitude. An increased adaptation to the higher light intensities may thus be expected for the surface plankton. Only a moderate increase is found, however, going from 65°N to 51°N. As the extent of the vertical mixing and the depth of the homogeneous surface layer also is of crucial importance for the light adaptation of the surface plankton, no simple correlation may be expected between latitudes and light adaptation of the surface plankton even at the same time of the year.

The curve 1 *a* in Figure 10 presenting the conditions in tropical surface water compared with the other surface curves gives on the other hand a significant proof of the hypothesis. The curve illustrating the tropical plankton is not strictly comparable to all of the other curves. Another light source — fluorescent light — was used. In principle, however, it makes no great difference. This is evident when comparing the curves 2 *a* (fluorescent light) and 3 *a* (incandescent light). The two series of measurements were made at about the same latitude during summer.

Unfortunately no photosynthesis light intensity curve of tropical shade plankton is available. Theoretically we must expect a curve practically identical with the curves 3 *b*, 5 *b* and 6 *b*. An increase in the temperature from 15°C to about 25°C may be considered to be of no real importance as the corresponding increase in temperature from 5° to 15°C had no significant influence; *cp.* p. 362. Finally, in the lowest part of the photic layer in the arctic and in the tropics the light conditions may be considered fairly similar if we disregard the differences in the length of the day.

In Figure 10 the curve 6 *b* (arctic shade plankton during summer) and 7 *a* (temperate surface plankton during midwinter) are identical. The temperature was practically the same (4.5°C and 6.3°C, respectively) and the average light intensity found during a summer day in the lower part of the

photosynthetic zone in the arctic is very likely about the same as found at the surface during a midwinter day at 56°N .

It has already been mentioned that the rate of photosynthesis at low light intensities theoretically cannot be considered to be strictly proportional to the concentration of active pigments. Nevertheless photosynthesis has been treated above as if such a strict dependency exists. In foliage leaves Gabrielsen 1948 showed that variations in the concentration of chlorophyll above 4 to 5 mg./dm.² do not affect the rate of photosynthesis at low light intensities. According to Beers law this also is to be expected theoretically. However, the problem is somewhat simpler in a dilute suspension of plankton algae, where the single chloroplast is not shaded by other chloroplasts.

According to Duysen and Huiskamp 1953 the single pigment molecules in a *Chlorella* cell is by no means equally illuminated. In the red part of the spectrum the molecules at the back of the chloroplast only receives 36 per cent. of the light illuminating the front of the chloroplast. Duysen and Huiskamp used laboratory reared *Chlorella* which have a chlorophyll content of 3 per cent corresponding to that in shade plankton; cf. Kok in Burlew 1953. In ordinary surface plankton collected outside the tropics — and in outdoor reared *Chlorella* — the chlorophyll content is only about half of that found in shade algae. According to Beers law, the coefficient of extinction is proportional to the concentration of the absorbent. Thus the concentration stands in the exponent. Accordingly, for cells containing only one half of the pigments found in Duysen and Huiskamp's cells, a single cell reduces the red light intensity to 60 %.

As the rate of photosynthesis is proportional to the light absorbed by the chlorophyll, a single cell of those containing only one half of the chlorophyll will thus have a rate of photosynthesis only $\frac{40 \times 100}{64} = 63$ per cent. of that of the others. Per unit of chlorophyll, on the other hand, the rate of photosynthesis in the cells poor in chlorophyll will be $2 \times 63 = 126$ per cent. of that in the others. It is in the same way possible to show that the rate of photosynthesis per unit of chlorophyll is 138 per cent. of that in the cells containing 3 per cent. chlorophyll, if the content is reduced to 0.75 per cent.

The value used above, 0.36 mg. C per mg. chlorophyll per hour at 1000 lux, was obtained from experiments with temperate surface plankton (about 1.5 % chlorophyll). In typical shade plankton (about 3 % chlorophyll) a value of 0.29 mg. C is better used ($0.29 \times 1.26 = 0.36$) and in surface plankton from the tropics (0.75 % chlorophyll) 0.40 mg. C per mg. chlorophyll per hour at 1000 lux ($0.29 \times 1.38 = 0.40$). In Figure 11 some of the typical curves from Figure 10 have been corrected accordingly. It must be stressed that the corrections may be considered only as a first approximation. The exclusive

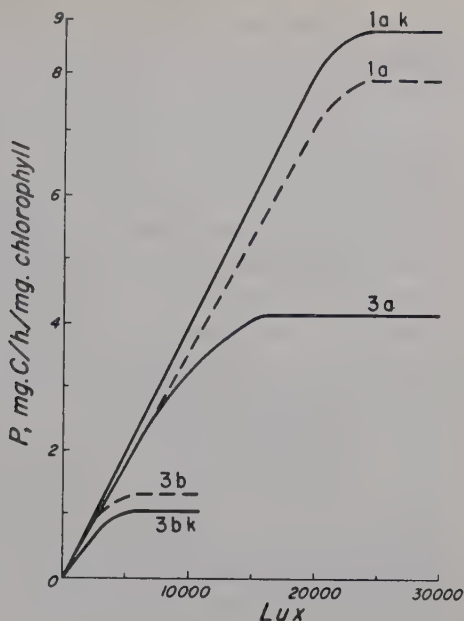


Figure 11. Selected curves from Figure 10, uncorrected (dashed lines) and corrected (full drawn lines).

use of red light for the computation is thus only one of several sources of error. The main purpose of Figure 11 is to show that the differences between the different curves in this way become somewhat — but not very much — greater.

4. The natural adjustment of enzyme concentrations in organisms

As mentioned in introduction the rate of an enzymatic process is a combined result of enzyme concentration and temperature. If the rate decreases due to a decrease in temperature, this may be completely counteracted by increasing the concentration of the enzyme. In Nature, organisms seem to be able to adjust their enzymes over a wide range at the level most appropriate to the prevailing ecological conditions. The ability of a single species to adjust the enzyme concentrations is, although by no means slight, of course often restricted. But taking Nature as a whole — by substituting the organisms with other species — the range of adaptation may be highly increased.

As according to current knowledge the majority of the proteins found in the protoplasm is identical with enzymes, the concentration of enzymes cannot increase to an unlimited extent. If as in bacteria the major part of the dry substance consists of protoplasmic protein — and thus enzymes — it is a matter of course that the growth rate at low temperatures cannot remain as high as at high temperatures. If the content of the enzymes in such a bac-

teria has to increase by a factor of say 2 in order to counteract a certain decrease in temperature, the content of total organic matter will also increase considerably. Accordingly the time for reproduction will not remain constant. Nearly twice as much organic matter must now be produced in order to reproduce the bacteria. Really rapid growth of bacteria is not very likely at low temperatures.

In other organisms where the enzymes do not constitute such a major part of the organic matter present, the total content of organic matter will increase only moderately by a considerable augmentation of the content of enzymes. In autotrophic plankton algae a major part of the cell constituents are found in the chloroplasts, connected with the photochemical partial process of photosynthesis. As the rate of photochemical processes are independent of temperature there is no need for increasing the responsible part of the cell at low temperatures. The increase in total content of organic matter at low temperatures due to an increase of the enzymes seems nevertheless occasionally to be of some importance in plankton algae. It is presumably no coincidence that by far the highest growth rate ever seen when cultivating unicellular algae was found when growing a special strain of *Chlorella pyrenoidosa*, 7—11—05, at 39°C (Sorokin and Krauss 1958). Nine doublings per day were reported. We are thus forced to assume that the effect of low temperature in plankton algae is not always completely counteracted in plankton algae by increasing the concentration of the enzymes. Nevertheless an approximate adjustment takes place.

Gessner 1949 has shown that it is possible to alter the assimilation number within a few days when transferring a plankton population grown at one depth to the light conditions found at another depth. As ordinarily at least, one cell division takes place per day such a rapid adaptation rate is easy to explain. If, on the other hand, the plankton is maintained in darkness for several days, no adaptation seems to take place. Talling 1957 found no marked modification of the light saturated photosynthetic rate in a culture of the freshwater diatom *Asterionella formosa* after 6 days in darkness. As no production of new cells takes place in darkness such a behaviour is readily understood.

The mechanism of adjusting the concentration of enzymes in plankton algae is by no means restricted to the enzymes active in photosynthesis. The rate of respiration in the algae is adjusted in the same way. Regardless of temperature and light adaptation the rate of respiration is nearly always about 5—15 % of light saturated photosynthesis; cf. Steemann Nielsen and Hansen 1959.

Algae being in a harmonious state adjust respiration to a rate matching the rate of photosynthesis. It must be born in mind that a harmonious coopera-

tion of the two processes is the background for a harmonious growth of the cells. Respiration is as necessary for growth as is photosynthesis! In terrestrial plants we find just the same. The rate of respiration is high in sun leaves matching the high rate of photosynthesis and low in shade leaves matching the low rate of photosynthesis. The rate of respiration as percentage of light saturated photosynthesis is practically the same both in sun and shade plants, both in plants from hot climates and in those from cold climates — measured at the temperature of the habitat. The same is also the case both in fast growing plants on eutrophic soil and in slow growing plants on oligotrophic soil (Stålfelt 1938).

The proper adjustment of the concentration of the enzymes determining the rate of respiration is found in lower animals as well. Several authors — e.g., Spärck 1936 — have given examples of cases in which the rate of respiration at the low temperature in arctic specimens was the same as measured at a much higher temperature in specimens from a temperate region. If the same temperature is used, the rate of respiration is much higher in the arctic animals compared to that of the same species from the temperate region indicating a higher concentration of the rate-limiting enzymes.

5. Absolute rates of photosynthesis and respiration in marine phytoplankton

In the above sections the rate of photosynthesis has been given either relatively or as a function of pigment concentration. When using the curves for describing the production pattern it is necessary to transform the curves to an absolute basis — *i.e.*, to present the rate of photosynthesis for example in mg. C per mg. C in the algae per time unit. If we know the ratio: active pigments to organic matter in the algae in all cases, such a transformation would be simple.

As we do not know the chlorophyll content in percentage of the organic matter in a single one of the experiments presented in Figure 10 we must estimate some likely values instead. The chlorophyll content in shade plankton from all latitudes has been put at 3 % of the organic matter. The chlorophyll content in indoor grown *Chlorella*, which must be considered as shade plankton, is as mentioned p. 364 just 3 %. The chlorophyll content in arctic surface plankton may very likely be estimated as 1.5 %, but 2 % would be as likely. The chlorophyll content in tropical surface plankton is probably extremely variable but generally lower than at high latitudes.

Some of the curves from Figure 10 — corrected in the same way as in Figure 11 — have been transformed in the above mentioned way and presented in Figure 12. In order to make clear the importance of the chlorophyll percentages chosen, two curves have been drawn for the tropical plankton

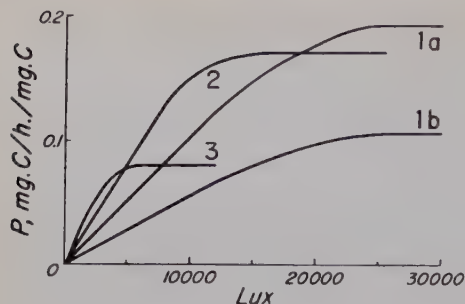


Figure 12. *Light intensity and rate of gross photosynthesis per unit of organic matter, 1=surface plankton from the tropics (a=1.0 per cent and b=0.5 per cent chlorophyll), 2=surface plankton from 69°N during summer (1.5 per cent chlorophyll), 3=shade plankton from 69°N or 50°N during summer (3 % chlorophyll).*

a) assuming that the percentage of chlorophyll is 0.5, b) assuming that it is 1.0 instead. Both percentages may very likely be found in tropical surface plankton, illustrating perhaps populations from respectively eutrophic and oligotrophic areas. But unfortunately it is a guess only.

Although the curves presented in Figure 12 must be considered with reservation, nevertheless some general features are obvious: a) the rate of photosynthesis measured per organic matter in the algae is rather independent of temperature in natural populations — Verduin 1957 arrived at the same conclusion when measuring photosynthesis per volume of algae — b) at low light intensities shade plankton has a higher photosynthetic rate per content of organic matter than does surface plankton. The rate of respiration in shade plankton per content of organic matter is low, however. In percentage of light saturated photosynthesis it is on the average about 8 (Steemann Nielsen and Hansen 1959). As the rate of light saturated photosynthesis, in contrast to the rates at low light intensities, is low in shade plankton, the absolute rate of respiration is also low. The compensation point therefore is found at a low light intensity, about 250—450 lux according to the curves presented in this article. Shade plankton is extremely well fitted for the life in the lowest part of the photic zone.

Due to the considerable rate of light saturated photosynthesis during summer at their normal habitat, surface algae can stand the high rate of respiration of about 10 per cent. of light saturated photosynthesis. However, as the photosynthetic rate at low light intensities is lower than in shade algae, the compensation point is found at a relatively high light intensity, about 600—1200 lux according to the curves presented in this article. The algae near the surface grow faster than those found in the lower part of the photosynthetic zone. Consequently the absolute respiration rate must be higher for the former. As already mentioned respiration must be considered as important as photosynthesis for growth. The latter process supplies only the necessary

basic organic matter to be used. Respiration on the other hand, by producing ATP, supplies the necessary energy for the many single processes taking place during growth.

Summary

A series of light intensity photosynthesis curves of marine phytoplankton is presented. The material includes curves of plankton from both the surface and the lower part of the photosynthetic layer, curves of arctic, temperate and tropical plankton, and curves both of summer and winter plankton.

A typical light adaptation of the plankton algae according to depth is found only if the watermasses within the photosynthetic layer are vertically stabilized thus preventing vertical mixing. A differentiation in "sun" plankton and "shade" plankton takes place.

In "sun" plankton the rate of light saturated photosynthesis computed per unit of chlorophyll is high. The compensation point is found at a relatively high light intensity, 600—1200 lux. In "shade" plankton the rate of light saturated photosynthesis per unit of chlorophyll is low and the compensation point is found at a low light intensity, 200—450 lux. Winter plankton from the surface in temperate regions behaves photosynthetically as typical shade plankton. According to all evidence the light adaptation is brought about simply by establishing a higher or a lower concentration of the enzymes active in photosynthesis.

The rate of light saturated photosynthesis being limited by an enzymatic process is a combined result of enzyme concentration and temperature. In arctic waters the concentration of enzymes is high compared with that at lower latitudes, both in relation to concentration of chlorophyll and in relation to the content of organic matter in the algae.

It is mentioned that the growth of bacteria does not seem to follow the general rule valid for plankton algae and most other organisms, namely that the effect of a low temperature at the habitat is counteracted by an increase in the concentration of the different enzymes. The background of this special behaviour is discussed.

Finally the rate of photosynthesis as measured per organic matter in the algae is discussed. It seems to be moderately independent of temperature in natural populations. At low light intensities shade plankton has a higher photosynthetic rate than surface plankton.

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Growth and Formation of Intercellularies in Root Meristems

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The well-known technique of growing excised roots in synthetic media under controlled conditions is sometimes a suitable method for the study of growth and nutrient problems. With wheat roots, employed in routine work at this laboratory, a convenient high degree of uniformity and reproducibility can be obtained in tests of short duration, up to 10—14 days. Infinite growth has not been attained with this plant (Almestrand 1956) and is not required either for many purposes. The uniformity of the longitudinal growth of the inocula corresponds to a standard deviation of the individual roots of about 25 per cent or less. With 24 roots in one experimental treatment, an error of the mean value of below 5 per cent is thus attained. If elongation is measured as elongation of individual epidermal cells (Burström 1957) a standard error in routine work of 2 per cent is easily attained. This is a highly satisfactory accuracy for most studies.

However, irregular exceptions from this uniform behaviour of the roots occur, in that individuals apparently randomly grow much more slowly and attain a structure differing conspicuously from the normal type both macroscopically and microscopically. They can simply be characterized and denoted as dwarfs. They form a group uniform in itself but with an elongation less than half the normal one. They are probably identical with the 'sinkers' described by Boll (1954 a, b), but since the identity is not clear, the provisory name of 'dwarfs' will be used. They should not be confused with genetically slow-growing roots of dwarf mutants in tomato (Lee 1958).

The problem of dwarf growth deserves attention for two reasons. Firstly,

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it forms a serious source of error in routine use of isolated root cultures. Even if it is obvious that the dwarfs represent an indubitably aberrant mode of growth such roots cannot simply be discarded. The number varies and it is obvious that it partly changes systematically with the culture conditions. Secondly, and even for the last-mentioned reason, the cause of the dwarf growth and the properties of the dwarfs deserve attention for their own sake.

The purpose of the present study was to find the causes of the dwarf growth of the roots, but it has extended into a study of the formation and contents of the root intercellularies.

Culture Methods

Dwarf growth has been observed for many years in wheat root cultures (Eroica wheat) under varying conditions. The standard nutrient medium usually employed has been a modification of the Almqvist solution (1956), containing KH_2PO_4 1/2500, $\text{Ca}(\text{NO}_3)_2$ 1/2500, MgSO_4 1/10000, Na_2SO_4 1/10000, Fe-versedate 10^{-6} M., thiamine 0.1, pyridoxine 0.5 mg./l., and glucose 1/20 M. Ten ml. of this solution in a 100 ml. flask are inoculated with 3 ca 7 mm. tips, cut from seedlings germinated in Petri dishes. The mode of germination of the sterilized seeds subsequently turned out to be a factor of importance for the appearance of dwarf roots. The cultures were kept at 22°C (or 25°C) in darkness or light (5000 Lux). The conditions of the material presented below vary somewhat, because part of it has been collected during one year from experiments designed for other purposes. Each conclusion drawn is based on repeated observations in several experiments not recorded here. Only examples are given in the tables. Single or uncertain notes of dwarf growth, not verified, have been entirely disregarded.

Under standard conditions dwarfs appear in a highly varying number, from 0 to 40 per cent, decidedly at random in flasks and between flasks of the same treatment.

Morphology of the Dwarfs

The dwarf roots are easily recognized, not only by the reduced length, but also by greater width and a yellowish translucent appearance, strikingly different from the whitish tinge of normal roots. This depends upon the fact that the intercellularies of the cortex ordinarily contain air. Under the microscope the intercellularies, owing to the light refraction appear black (Figure 1), extending in the meristem almost to the very tip of the root where they converge. *In the dwarfs, however, the intercellularies are entirely filled with water.* This can be seen in a microscope, and the same yellowish, translucent colour can be obtained by slightly squeezing the air out of a piece of a root. It seems that this water-filling of the intercellularies is the most important



Figure 1.

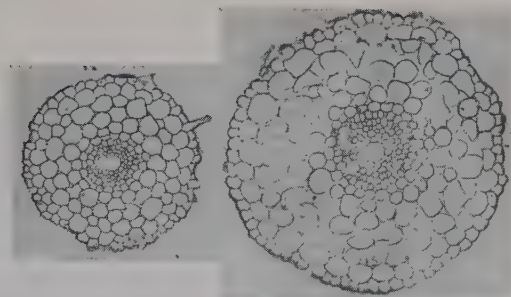


Figure 2.

Figure 1. *Normal and dwarf roots.* Note the striation of the normal root (A), owing to dark intercellularies converging towards the tip, and the translucent appearance of the dwarf roots (B).

Figure 2. *Transverse sections of normal and dwarf roots.* Note the large cortical intercellularies in the dwarf root.

property of the dwarfs. The morphological characteristics have been assembled in Table 1.

The microscopic organization is exemplified in Figure 2 and Table 2 (from different experiments). The picture illustrates thickening of the root, transverse growth of all parts, particularly the cortical parenchyma. Far from being diminished by the slightly hypertrophied cells the intercellularies are conspicuously large, especially in the middle part forming a very loose tissue. Histologic details are recorded in Table 2. Disregarding the mode of production of the dwarfs, which will be dealt with in the next section, we may conclude from the data: (1) The dwarf roots are shorter and thicker with some, but not much reduced root volume; (2) in transverse section the number of cortical and endodermal cells remains unchanged; (3) the number of epidermal cell rows is increased but the number of cells in each row decreased, usually resulting in no change in the frequency of cell divisions; (4) the cells are, on the other hand, much shorter in the spontaneous dwarf roots.

Table 1. *Morphological differences between normal and dwarf roots.*

	Normal	Dwarfs
Size (cf. Figure 1 and Table 2)	long, slender	short, stunted
At low magnification	whitish, opaque	yellowish, translucent
Under the microscope ...	all parts except epidermis dark owing to refraction in air-filled intercellularies	cortex translucent, all intercellularies water- filled

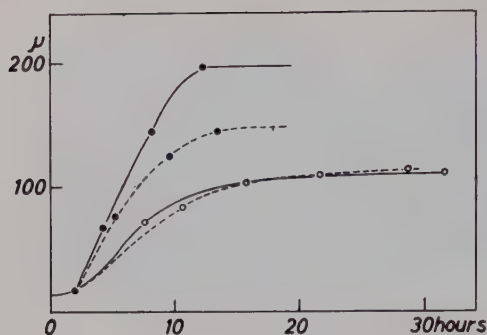


Figure 3. The time course of the cell elongation of normal and dwarf roots in light (5000 lux white light) and darkness. ●— normal, dark grown, ●----- normal light, ○— dwarfs, dark, ○----- dwarfs light.

The true course of cell elongation has been determined (Burström 1957) and plotted in Figure 3. The rate of elongation is considerably reduced, but the duration of the elongation usually extended. Summarizing, it may be said that in the dwarfs the meristems function normally only with some shift in the directions of the divisions of the epidermis, radial growth is much increased, but elongation slow although extended in time, leading to a more or less, often considerably reduced root length.

Conditions Favouring Water-Filling

Spontaneous water-filling is an irregular phenomenon. It is, however, more common under certain conditions. It is regularly connected with auxin-inhibition of the elongation (Table 3) and Ca-deficiency (Table 4). Table 3 is particularly interesting, because it shows a gradual shift from normal to dwarf roots with increasing auxin, but both types of roots respond to auxin

Table 2. The influence of germination conditions on the formation of dwarf roots. Roots developed in air or submerged prior to inoculation. Darkness, 7 days. Roots sectioned and studied microscopically.

Germination conditions	Number of roots		Growth mm.	Root diameter μ	Stele diameter μ	Epidermal cells		Number of cells in transverse section			Total number of epidermal cells per root
	Normal	Water-filled				Length μ	Breadth μ	Epidermis	Cortical parenchyma	Endodermis	
Aerial roots	32	4	41.5 ± 1.2^1	318 ± 2	126 ± 2	275 ± 4	16 ± 0.3	69 ± 1	101 ± 1	22 ± 0.7	10,400
Wet roots	4	32	15.1 ± 0.8^2	467 ± 11	178 ± 3	122 ± 3	18 ± 0.6	84 ± 1	106 ± 3	27 ± 0.4	10,400

¹ Normal roots; water-filled roots 19 mm.

² Water-filled roots; normal roots 29 mm.

Table 3. *The influence of auxin on the formation of dwarfs. Darkness, 5 days.*

1-NAA M	Number		Length mm.		Epidermal cell length μ	
	Normal	Dwarfs	Normal	Dwarfs	Normal	Dwarfs
0	22	6	24 ± 1	9 ± 1	215 ± 5	178 ± 4
10^{-7}	19	16	15 ± 1	6 ± 1	175 ± 4	146 ± 3
$3 \cdot 10^{-6}$	8	25	8 ± 0.5	3 ± 0.2	108 ± 2	87 ± 3
10^{-6}	0	36	x	1	x	x

in practically the same way. This is less clear with Ca, but Ca-deficiency cannot be graded in the same manner.

Light, on the contrary, does not affect the water-filling; very many experiments including a comparison between light and darkness have failed to disclose a regular light effect, even at 40 % light inhibition of the elongation (Table 4, 6, Figure 3).

Nevertheless, the question must be raised whether auxin and Ca-deficiency cause water-filling and this leads to growth reduction or whether they reduce growth and this causes a water-filling. The second possibility is *a priori* more likely, and can be tried experimentally. On the other hand, the mentioned identical growth inhibition by auxin in normal and dwarf roots would rather imply that water-filling is something independent of the growth-inhibiting action.

Several types of experiments were carried out in order to find an answer to this question. — After excision of roots and inoculation of flasks these were evacuated until no more gas escaped from the roots (Table 5). Evacuation produced water-filling and dwarf growth, at least superficially similar to the spontaneous dwarfs.

An objection may be made that evacuation deprives the solutions of oxygen, but this is done already during autoclaving of all solutions. The shallow, 5 to 6 mm. layers of solution ought to regain equilibrium with the air rather soon.

As the next possibility the germination conditions were studied. In a Petri dish roots may grow in the air or close to the wet filter paper more or less

Table 4. *The influence of Ca and light on the formation of dwarfs. 6 days.*

Ca M	Dark				Light			
	Number		Length mm.		Number		Length mm.	
	Normal	Dwarfs	Normal	Dwarfs	Normal	Dwarfs	Normal	Dwarfs
0	2	15	53	26 ± 1	0	15	x	21 ± 1
$2.5 \cdot 10^{-4}$	17	1	55 ± 2	24	16	1	33 ± 2	21
10^{-3}	18	0	35 ± 1	x	16	2	32 ± 2	19

Table 5. *The influence of evacuation and high Ca-concentration on the formation of dwarf roots. Darkness, 6 days.*

Treatment	Number of roots		Growth mm.	
	Normal	Dwarfs	Normal	Dwarfs
Control ¹	23	11	64 ± 1	26 ± 3
+ CaCl ₂ 2 · 10 ⁻³ M	32	4	44 ± 2	12 ± 4
+ CaSO ₄ 2 · 10 ⁻³ M	34	2	60 ± 1	19 ³
Roots evacuated ²	1	19	62	18 ± 1

¹ With Ca 10⁻⁴ M. — 2 roots injured and dead.

² After inoculation evacuated in a vacuum desiccator until no more gas was given off from the roots. — 16 roots had their meristems injured (in evacuation?) and dead.

³ 11 and 27 mm. respectively.

submerged, or at least clearly wet. This increases dwarf growth to above 50 per cent in many instances (Table 2, 6). However, it was established that the roots of wet-grown seedlings contain gas-filled intercellularies, just as in the aerial roots, but they must lose the aerial system rapidly following inoculation. Roots were thus simply washed before inoculation. After excision they were suspended in a large amount of distilled water, left for two hours under occasional shaking and then transferred to the culture flasks (Table 7). Washing significantly increases the number of dwarfs.

As a practical remedy against dwarf growth gentle rocking of the root cultures on a shaking table was tried (amplitude 5 mm. horizontally, 50 times a minute). This reduces considerably the formation of dwarfs, perhaps by facilitating the aeration, but also causes in itself a decrease in root growth. This is exemplified by the following experiment (10 days' duration, darkness, 16 roots):

	Root length mm.
Standing cultures	66.2 ± 3.3
Shaken cultures	37.4 ± 1.1

Table 6. *The influence of germination conditions and light on the formation of dwarf roots. Pretreatment as in Table 3. Experiments in darkness and with 5000 lux, 7 days.*

Treatment	Number of roots			Growth mm.		Epidermal cell length μ	
	Normal	Water-filled	Dead	Normal	Water-filled	Normal	Water-filled
Aerial roots, darkness	17	0	1	54 ± 2	x	287 ± 7	x
Submerged roots, darkness	4	12	2	51 ± 3	11 ± 1	x	102 ± 2
Aerial roots, light	18	0	0	30 ± 2	x	137 ± 2	x
Submerged roots, light	2	10	6 ¹	27	10 ± 1	x	111 ± 3

¹ 3 roots (1 flask) owing to infection.

Table 7. *The influence of washing the roots before inoculation.* Roots after excision suspended for two hours in distilled water, then transferred to culture solutions. Two experiments. Darkness, 4 days.

Treatment	Number of roots			Root length mm.	
	Normal	Dwarfs	Dead	Normal	Dwarfs
Controls	28	2	0	37 ± 0.5	(21)
Washed	17	9	4 ¹	34 ± 1.0	24 ± 2
Controls	26	3	1	34 ± 0.5	17
Washed	19	11	0	33 ± 1.0	19 ± 2

¹ 1 flask (3 roots) probably owing to some contamination.

The roots are apparently remarkably sensitive to mechanical influences, even if they never touch the walls of the flasks. Whether this should be called a seismic or a rheic reaction is unknown. The results indicate that insufficient aeration may produce dwarf growth, but this may be due either to better oxygen supply or removal of carbon dioxide. The growth properties of the stirred roots must be left for further studies.

The Composition and Origin of the Intercellular Gas

It is known from the literature (Laing 1940, Conway 1937) that in intact plants the internal atmosphere of subterranean parts consists almost constantly of 79 per cent N₂ as in other plant parts (see, *e.g.*, Wardlaw and Leonard 1936, 1940, Hackney 1943, Burton 1950) but with CO₂ amounting to (2—)5—16 per cent and correspondingly less O₂. In water plants the O₂ and CO₂ contents of rhizomes depend upon the photosynthesis of aerial parts (Laing 1940), and Conway (1937) has shown with *Cladium* that O₂ is actually delivered mainly through the intercellularies to the roots. This is also indicated by Jensen (1957) finding the surprisingly high RQ of 4.3 in intact root systems of maize.

The composition of the gas phase in roots grown excised is virtually unknown. A high CO₂ content is to be expected, but microscopic tests have led to a surprising conclusion.

Excised roots or tips of such roots were brought from the culture solutions to microscopic slides under cover glass, still in the same solution and without contact with the air. If a solution of KOH was added to the margin of the cover glass and allowed to spread to the roots, the intercellularies rapidly disappeared, particularly from the meristems and adjacent part. This can easily be followed under the microscope; it has been verified repeatedly, it never

fails, but it cannot be recorded in pictures. The following notations from one test may be quoted as an example:

Root tips, 1 mm. (meristem only) with KOH added. Most intercellularies disappear instantaneously, contracting from one or both ends; no microscopically visible remains after a few seconds. Single intercellulary channels remain apparently unchanged, strong menisci against the walls; they suddenly start contracting, subsequently disappearing completely. It looks as if KOH did not easily enter all parts in the root interior. After 15 minutes no intercellularies left.

The same picture was obtained with tips from roots grown in ordinary water cultures, also with older parts of excised root but not with mature parts of intact roots (more than 1 cm. from the tip). Their intercellulary gas did not dissolve in alkali.

Although the test is strictly a qualitative one, the results are so clear-cut, that the conclusion must be drawn that *the internal gas phase of the root meristems consists of practically pure carbon dioxide*. The same holds true of also older parts of excised roots. Unfortunately the amounts of gas in the tips are too small and too difficult to remove for a chemical determination by any available method.

The origin of the intercellulary gas must be the respiratory carbon dioxide. Thus it may be argued that a low respiration may lead to lack of gas-intercellularies and that the dwarfs owing to their bulky shape have a low respiration per volume. In order to test whether this could explain the water-filling, determinations have been made of the respiration of the roots.

The Respiration of the Roots

The respiration of our standard root material has been thoroughly determined in several investigations, and the following data may be quoted from material of E. Nyberg:

A 10 mm. root tip, volume 1.6 μl , respire under standard conditions exogenously on an average about 1.8 μl . O_2 per hour. It grows about 5 mm. in length, or 0.8 μl . in volume per 24 hours. The tip thus consumes at least 15 μl . O_2 on 0.8 μl . increase in volume per 24 hours. The RQ has been determined by Karlsson and Eliasson (1955). The tip has an endogenous RQ of about 0.85, with exogenous respiration of 0.92 in short time tests. The difference between O_2 and CO_2 is about the same in both instances or 1.2 μl . per root tip and 24 hours.

A similar computation can be made on the respiration data in Table 8. Thus during growth the volume of CO_2 produced is at least 100 times the volume of newly formed intercellularies, and even a theoretical retention of

Table 8. *Respiration of normal and dwarf roots.* Roots during germination in air or water, grown for 6 days in darkness before respiration determinations manometrically in Warburg-Barcroft apparatus.

Type of roots	Number of pieces	Length mm. ± 0.4	Width mm. ± 0.01	Weight mg.	Volume mm. ³	Surface mm. ²	Respiration O ₂ µl/h.		
							Total	per mm. ³	per mm. ²
Normal roots	16	11.3	0.35	14	17.3	198	31.3	1.81	0.158
	16	11.1	0.34	13	16.3	191	30.1	1.85	0.157
Dwarf roots	13	10.3	0.46	20	22.3	194	34.1	1.53	0.176
	12	9.9	0.48	21	21.8	180	34.0	1.56	0.189

Pretreatment during germination	Number of roots	
	Normal	Dwarfs
Roots in air.....	25	4
Roots in water ...	9	21

Type of roots	Growth mm.	Epidermal cell length µ
Normal roots	58 ± 1	213 ± 4
Dwarf roots.....	17 ± 1	149 ± 4

CO₂, assuming a true RQ=1, exceeds the volume of their intercellularies by a factor of about 10. Solubility and permeability conditions permitting, the gas production is more than adequate for maintaining the internal gas phase.

With dwarf roots three series of respiration determinations — one of which is recorded in Table 8 — have been carried out. They disclose that the respiration of the dwarfs is only slightly lower than that of normal roots on a volume basis (or weight basis) and equal or higher on a surface basis. These differences alone can hardly explain the lacking internal gas phase.

Table 9. *The growth of normal and evacuated roots under an oxygen atmosphere.* For method of evacuation cf. Table 5. In B and D a slow current of sterile filtered pure oxygen was passing over the surface of the culture solutions without stirring the solutions and without bringing the roots in contact with the gas phase.

Treatment	Dwarf		Normal roots		"Typical" roots: normal roots of A, B, and D, dwarfs of C		
	Number	Length	Number	Length	Diameter mm. ± 0.01	Volume growth per root mm. ³	Epidermal cell length
A Control roots, air.....	4	17 ± 3	16	49 ± 2	0.41	6.3	203 ± 5
B " oxygen ...	0	x	21	32 ± 1	0.34	2.8	177 ± 4
C Evacuated roots, air ...	16	16 ± 0.5	4	43 ± 2	0.50	3.2	150 ± 4
D " oxygen	2	19	17	28 ± 1	0.31	2.1	167 ± 5

Intercellularies in the tips:

A and B normally developed gas intercellularies, gas soluble in KOH,

C dwarfs: water-filled, normal roots: resembling A

D dwarfs: water-filled, normal roots: 2 roots with gas intercellularies, gas soluble in KOH, the rest water filled.

Nevertheless, experiments were carried out with an increased oxygen tension in order to compensate the decreased oxygen uptake in the bulky dwarfs (Table 9). An oxygen tension in equilibrium with pure oxygen is supra-optimal for roots (cf. Eliasson 1958) in the present instance reducing the volume growth to one half the normal value, but the roots are thin with no dwarf symptoms although the intercellularies are water-filled. The conspicuous result is that *the stunted dwarf growth is prevented by oxygen, but the gas intercellularies are not restored.*

Thus we have separated dwarf growth from lack of internal gas phase, and a discussion of this whole morphologic complex is rendered possible.

Discussion

The Formation of Intercellularies

It has been established that under all ordinary culture conditions, even in water cultures, the wheat roots contain gas in the intercellularies and these extend into the meristem, reaching to about 50 or 100 μ from the apex proper (boundary between meristem and calyptra). Water-filling of the intercellularies is decidedly an abnormal condition, in our experience occurring only in excised roots, never in intact plants.

Discussing theoretically the histologic meaning of 'free space' Briggs and Robertson (1957) assume this to consist of injected intercellularies, wet cell walls, and part of the cytoplasm. It seems pertinent to point out that with ordinary tissues, even of submerged organs, the intercellularies ought to be excluded from computations of the volume normally available for free space uptake of solutes.

The most interesting result is that the intercellularies of the meristems contain pure carbon dioxide and that water-filling of these does not impede their formation. They are on the contrary exceedingly large when filled by solution instead of carbon dioxide. It is for this reason necessary to distinguish between *the formation of intercellularies* and *the excretion of gas* to the cavities.

The water-filling, or more correctly lack of gas excretion, is obviously not caused by the dwarf growth, since it appears also in the slender roots grown under an oxygen atmosphere. It has also been observed that lateral roots although thin may entirely lack internal gas phase.

Intercellularies are initiated already in the meristem, where they attain the shape of thin lamellae between the longitudinal rows of cells in the periblem; they continue in the well-developed cavities in the mature cortex. The intercellularies grow by apical elongation, and at least in the apices they contain pure carbon dioxide. The secretion of this gas between the cells into the intercellularies or during their formation must depend upon the production of

carbon dioxide in the respiration. Only a high CO_2 tension is by itself no reason for the separation of a gas phase, because CO_2 in the first hand ought to escape outwards by diffusion; the diffusion potential between the root tips, where subsequently a pure CO_2 -atmosphere can arise, and the external solution in equilibrium with the air is considerable, and the distance from the centre of the periblem or cortex to the surface not more than 50 to 80 μ . Certain fairly specific conditions must then be fulfilled for the excretion of an internal CO_2 -atmosphere. It should be pointed out that Lundegårdh and Burström (1933) from the rate of CO_2 -exudation concluded that the CO_2 -pressure in the root must exceed 1 atmosphere. Thus the *permeability resistance* to CO_2 ought to be a factor regulating the internal tension. It might be advocated that Ca-deficiency and auxin excess increase the permeability thus favouring the exomosis of CO_2 , decreasing or preventing an internal gas exudation. Ca-deficiency does not cause more than about 30 per cent reduction in the respiration corresponding to the reduction in amount of mitochondria (results of E. Nyberg and K.-L. Lindblad: in preparation for publication). It is hardly likely that this can cause such a decrease in the CO_2 tension that no gas is given off to intercellularies.

More important is probably that there must be some reason for the formation of gas cavities at all. An intercellulary is formed by the separation of neighbouring cells. The walls are imbibed by water and if the water supply is unhampered in the free wall space, or the permeability of the cells great, it is easy to imagine that water is continuously flowing into the gradually growing intercellular cavity. It is more difficult to understand why gas spaces are ever formed, even if the CO_2 tension in the cytoplasm is high. We come to the conclusion that the exudation of gas internally must be governed by the CO_2 tension owing to the respiration, the permeability for the uptake of water, and the permeability for the output of CO_2 . The result is not easily predicted in each specific instance.

The formation of gas interstices must be facilitated if there are already pre-formed gas spaces in contact with the sites where the intercellularies should be formed. CO_2 must then be given off to the gas space till equilibrium, and with the space increasing during the growth of the cells, CO_2 is continuously given off. Normally the intercellulary is formed as a *continuum*, and it meets with large resistances for a submerged tissue to form a gas space *de novo*. Since the only gas available in the growing tip is CO_2 , it explains why the apical ends of the intercellularies contain only this gas, or practically so. These assumptions are not too far-fetched and they explain why evacuation causes disappearance of the gas spaces in the growing, newly-formed tips: the initially present gas spaces become water-filled, and a new formation of gas spaces in the tip is very much impeded or simply prevented.

It is difficult to decide whether a reduced respiration in itself is sufficient for causing a water-filling or preventing a gas-filling of the intercellularies. This depends upon how great the tension of CO_2 is. With no preformed gas spaces not even an oxygen atmosphere will suffice for producing new ones, but, on the other hand, shaking practically prevents water-filling, which indicates that the aeration is not without importance. Pre-washing of the roots most likely removes air bubbles from the root surface and root hairs, causing the inocula to sink. The results show that this does not necessarily prevent gas space formation, but it is greatly inhibited. Since formation or non-formation of gas spaces depends upon the reaction of the root pieces immediately upon inoculation in the nutrient medium, it may be assumed that such a treatment slows down the respiration temporarily, the newly formed intercellularies become water-filled, and once this has happened the roots have difficulties in restoring the gas phase again, as outlined above. In any case, the respiration and permeability conditions in the freshly started cultures are probably of decisive importance for the future mode of intercellularly formation.

The Cause of Dwarf Growth

No final explanation can be given of this phenomenon either, but some possibilities should be mentioned. It can be separated from water-filling of the intercellularies. Dwarfs are always water-filled, but water-filling does not necessarily lead to dwarf growth, *e.g.* if the oxygen supply is high. Thus it might be concluded that water-filling leads to low respiration, and this in its turn to dwarf growth. The following two points should then be considered.

(1) There is no immediate reason why water-filling should decrease the respiration, since the intercellularies contain not air but CO_2 . It is hardly possible that the intercellularies extending longitudinally should facilitate a radial transport of O_2 either. (2) The respiration per volume is not much lower in dwarfs than in normal roots, and per surface unit it is virtually the same. However, owing to the bulky shape the respiration in interior layers may be much lower, and it is probable that the root growth is governed by activity in interior cell layers (Burström 1949, Burström and Hejnowicz 1958).

Whatever the immediate cause of the dwarf growth is, it leads to a regular inhibition of the elongation under lateral swelling and formation of large intercellularies. This inhibition does not appear in roots grown in the light; but light-grown roots show no increased lateral growth. The mechanism of light inhibition of root growth is not well understood; its action spectrum is known (Kohlbecker 1957) and it has been tentatively connected by Hejnowicz (1958) with the chlorophyll system. It should be mentioned that dwarf

roots form chlorophyll abundantly, histologically on the normal pattern (Burström and Hejnowicz 1958). The connection between dwarf growth and light seems to be of the nature that the same part of the cell elongation mechanism is inhibited in both cases but in different ways, which could explain the remarkable similarity between the course of elongation in dwarf and light roots (Figure 3), and the light insensitivity of the dwarfs.

On the other hand, auxin inhibition is independent of the dwarf growth. The time course of auxin inhibition of the cell elongation is well known from previous studies (cf. Burström 1957). It implies a rapid start of the elongation and an abrupt cessation. The light and dwarf inhibitions are distinctly different: a normal start and subsequent slowing-off of the elongation.

Both criteria — the interaction between the growth factors and the time course of the inhibitions — justify the conclusion that the light and dwarf inhibitions are independent of an auxin regulation of the elongation. It is worth mentioning that recently Blaauw-Jansen (1959) has tentatively connected light action on longitudinal growth with gibberellins.

The CO₂ Retention in the Meristems

The exudation of CO₂ to the intercellularies of the meristems require some comments. The RQ of the tips is known to be below 1 in these roots, as well as in others. It can be computed from data of Karlsson and Eliasson (1955) that the difference between O₂ consumed and CO₂ actually given off under certain standard conditions amounts to 1.2 μ l. per 0.8 μ l. increase in root volume, regardless whether the roots respire endogenously (RQ 0.85) or exogenously (RQ 0.92). Some of this CO₂ must be withheld in newly formed intercellularies, some should remain dissolved in or adsorbed on the cytoplasm. Haber and Brassington (1959) state that tissues in this way accumulate more than their own volume of CO₂. Thus this retention is sufficient for explaining the low RQ values in the meristems, even with a true RQ=1, and no special kind of metabolism must occur (Ruhland and Ramshorn 1938, Karlsson and Eliasson 1955). It is always tacitly assumed in respiration determinations that the CO₂ given off equals that produced, or nearly so, but in growing root tips this obviously cannot hold true.

Summary

Wheat roots grown in air or nutrient solution, intact or excised, contain gas-filled intercellularies, extending far into the meristems. At the tips these contain pure CO₂.

In excised grown roots the gas spaces may disappear and the intercellularies become filled with water. This is favoured by a low Ca content, auxin addition, and pre-washing of the roots before the inoculation. The mechanism of intercellular formation and the appearance of the gas spaces is discussed, and it is concluded that respiration and permeability conditions are of decisive importance.

Water-filling usually leads to an inhibition of the elongation and a 'dwarf' growth resembling the 'sinkers' of Boll (1954 a, b). The respiration of these dwarfs is not much lower per volume and the same per surface as in normal roots. Dwarf roots are insensitive to light, otherwise inhibiting growth. The time course of growth is the same in dwarfs and light inhibited roots. It is concluded that dwarf growth and light inhibit the same part of the elongation mechanism. This is independent of the auxin inhibition of elongation.

The retention of CO_2 in the growing tips is discussed with reference to the low RQ of these parts.

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The Influence of Gibberellic Acid on the Transaminase Content of Germinating Barley Seeds

By

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From earlier investigations it is known that plants treated with gibberellins show higher enzyme activity than plants not treated (8). The number of enzymes investigated is, however, small. For germinating barley Munekata and Kato (5) found that the activity of α - and β -amylase, catalase and protease was increased. Recently Sandegren and Beling (7), also investigating germinating barley, demonstrated that the activity of α -amylase and cellulase was increased greatly. The increase in the protease activity was smaller although evident.

As the nitrogen metabolism is likely to be accelerated by treatment with gibberellic acid it should be interesting to study whether enzymes engaged in nitrogen metabolism are influenced by gibberellic acid treatment. In the present paper we have studied the influence of this compound on a transaminase.

Rather little is known concerning the occurrence of transaminases in plants (1, 2, 9). The existence of transaminases in plants is however certain. Recently Cruickshank and Isherwood (3) determined the amounts of glutamic-oxalacetic-transaminase (GOT) and glutamic-pyruvic-transaminase (GPT) present in wheat germs.

Germinating seeds of barley were used in our experiments and the occurrence of GOT was investigated as this transaminase is relatively easy to determine. Studies were made on the GOT content in the seeds when treated with different concentrations of gibberellic acid.

Methods

Barley, strain Hertha, harvested 1958 was used for the investigation. Eighty g. of barley seeds (with 87.3 % dry matter) were soaked at 25° for 40—46 hours in 120 ml. of water or in 120 ml. of solutions of gibberellic acid¹ at different concentrations. After soaking, the barley seeds were transferred to glass containers kept at 25° and moist air was blown through the containers for 24 hours (for further details see Nielsen and Bergqvist (6)). At this stage the seeds had germinated, the roots and the coleoptiles being visible. The seeds were dried for 3 hours at room temperature and ground. The GOT was extracted according to the method described by Cruickshank and Isherwood (3). The dried powder was extracted twice. The amount of GOT found by the second extraction did not exceed 20 % of the amount found by the first extraction and a third extraction was thus considered to be of no importance. The determination of GOT was carried out according to Karmen (4). The Karmen definition of a GOT-unit was used. A Kabi reagent (obtainable from AB Kebo, Stockholm) was used in the determinations.

Results

The first experiments were performed in December 1958 and January 1959, a few months after the harvesting of the barley. In ungerminated barley seeds the amount of GOT-units per g. of dry matter was about 20. When the barley has germinated for 64—70 hours (soaked for 40—60 hours in water and aerated for 24 hours) the content of GOT was somewhat greater. The determinations gave figures varying between 20 and 25 GOT-units per g. of dry matter, the losses in dry matter during the germination not being taken into consideration. Only a slight increase in the amount of GOT thus occurred during germination.

If, however, the seeds were soaked in solutions of gibberellic acid a much greater increase in the amount of GOT was found. When soaked in a 3 ppm. solution the GOT-units raised to 40, when soaked in a 10 ppm. solution to 65 and when soaked in a 30 ppm. solution to 70. The maximal effect is obtained with a 30 ppm. solution. The treatment gibberellic acid thus gives rise to a strong increase in the GOT-content.

Earlier (6) the influence of gibberellic acid upon the respiration of barley seeds was investigated. The same strain of barley and the same method for the germination was used. The experiments were performed a few months after the harvest. The maximal effect was obtained with a solution containing 10 ppm. gibberellic acid, the respiration being about twice as intensive as with water. The influence of gibberellic acid on the GOT-content of the barley seeds thus seems to be stronger than the influence on the respiration

¹ The gibberellic acid used was kindly supplied by Merck Sharp and Dohme, Research Laboratories, U.S.A.

When the experiments were repeated in February and March 1959 it turned out that the GOT-amount present in the seeds was markedly higher. The seeds still responded to a gibberellic acid treatment but the increase in GOT was relatively smaller. The concentration of gibberellic acid afforded for the maximal effect was also smaller. Whereas the amount of GOT-units in seeds soaked in water was about 50, the amount was 70 when the seeds were soaked in a 0.3 ppm. solution and 100 when soaked in a 1 ppm. solution.

The stimulating effect of gibberellic acid treatment on the respiration was correspondingly altered by the experiments in February and March. The effect of the treatment was smaller and the concentration needed for maximal effect was smaller, about 1 ppm.

The reason for the different behaviour of fresh and more mature barley seeds is not known. One explanation would be that the amount of gibberellic acid present in the seeds is increasing with the ripening. This would explain the smaller response to the gibberellic acid treatment and the fact that smaller concentrations are afforded for the maximal effect.

The possibility that the effect of the gibberellic acid was produced by a stimulation of the enzyme system was examined, and it was shown that the addition of gibberellic acid to the enzyme system had no stimulatory effect. The increase in the GOT-activity found must thus be considered as a real increase in the amounts of GOT present in the barley seeds.

Some preliminary experiments have been performed concerning the content of GPT. It seems that the content of this transaminase is also increased by treatment with gibberellic acid.

The marked increase in the GOT-content following gibberellic acid treatment makes an analytical application possible. Some broths from gibberellin fermentations were tried and results obtained which were in very good agreement with the results obtained with the respiration method (6). The method using determination of the GOT-content is applicable for solutions having concentrations from 0.3 to 10 ppm., depending on the age of the barley seeds employed.

Summary

When freshly harvested barley seeds are soaked in solutions of gibberellic acid having concentrations of 1 to 30 ppm. and then allowed to germinate, the content of GOT is greatly increased as compared to that of barley seeds soaked in water. After soaking in 10 or 30 ppm. solutions the content of GOT is more than twice the amount of GOT in seeds soaked in water, provided the barley seeds are used some few months after the harvest. 4 to 5 months after the harvest the response is smaller and besides the content of GOT in seeds

soaked in water is greater. Also the concentration of gibberellic acid needed for the maximal response is smaller, about 1 ppm.

The content of GPT also seems to be increased when the seeds are treated with gibberellic acid.

The method described is applicable for a biological analysis of gibberellins.

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Changes in the Transpiration of Wheat Leaves Caused by Changes in the Properties of the Root Medium

By

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Introduction

It has been shown repeatedly (1, 3, 8, 9, 14) that the transpiration of leaves can be rapidly increased or decreased. Changes of this type can be recorded within 15—30 seconds and have been found mainly after excision of leaves. An increase of the transpiration is obtained when the cutting is undertaken in air and it is well known that such an excision of a leaf is followed by an opening of the stomata (4). Both these phenomena can be understood as caused by an increase of the supply of water to the leaf as the stomatal movements are controlled by the water relations of a plant as well as by the light intensity (1, 14, 16). The increase of the water supply is explained as a consequence of a sudden release of the stress, normally present in the water columns in the conducting elements of a transpiring plant (1, 9).

The transpiration is decreased when the excision is undertaken with the place of cutting immersed in water. This has been explained as a consequence of a sudden uncoupling of the "root pressure". Whether this decrease of the transpiration is connected with a closure of the stomata is not known. However, Virgin (22) has assigned most of the changes in the output of water from the leaves, which can be recorded in a continuous registration of the transpiration, to changes in the width of the stomata and he assumes that the transpiration rate is directly related to the stomatal aperture. Reservations

were put forward in the case of a rapid decrease of the light intensity, where temperature effects were assumed to be involved. Spanner (15) has claimed that in altering the linear dimensions of a system, diffusion is changed in the ratio of the square of the scale factor. "For a leaf 5 cm. diameter with stomata of $25\ \mu$ the stomatal diffusion shells arise $(5 \times 10^{4/25})^2$ or four million times as fast as the leaf shells. They are much less affected by winds therefore and it is obvious that the stomata will be main factor controlling transpiration whenever air movement is sufficiently rapid". Thus the changes in transpiration can presumably be attributed to the stomatal transpiration. Whether this can be changed without a change in the width of the stomata is not known but seems to be rather unlikely in the case of such rapid changes as those described here.

The suggestions of a close connexion between the transpiration rate and the stomatal aperture and of a similar connexion between transpiration and water supply to the leaf, involve that a rapid response to changes in the water supply would be performed by the stomata. Contradictory to this Virgin (22) has adopted the idea that the uptake of water to the guard cells takes place rather slowly and that this is the limiting factor in the stomatal opening process. However, the conclusions drawn by Virgin seem to the author to be somewhat questionable. Virgin (22) has summarized his main results on page 301. "The transpiration continues to increase for a few minutes in darkness given in the middle of the opening phase in light. This indicates that the formation in light of osmotically active substance is a rapid process, as the time lag is presumably due to the diffusion of water into the guard cells." The reason for this presumption is found on page 287. "As the photosynthetic mechanism begins to work immediately after the onset of illumination there is no reason to assume a delay in the formation of osmotically active substance." Disregarding the facts that this has a taste of arguments in a circle and that induction phenomena have been shown to be involved in photosynthesis (for ref. see Vejlby 1958) it can hardly be assumed that a diffusion pressure deficit great enough for a water uptake can arise instantly even though the photosynthetic mechanism has begun to work.

It seems to the present author that Virgin's results do not contradict the assumption that the change in the amount of osmotically active substance is the slower process while water transport takes place faster.

From a theoretical point of view Spanner (15) has concluded that an evening out by diffusion of concentration differences takes place very rapidly in cells. He has compared the dimensions of in vitro experiments where the distances are 1—10 cm. with those of cells where distances of the order 1—10 microns are involved. He finds that with the dimensions quoted the process

takes place one hundred million times as fast in the cell. Thus the diffusion of water in the guard cells would presumably be a rapid process. The possibility of a metabolic component in the water uptake must also be kept in mind (17). Therefore, no obstacles are present to the assumption that changes in the water supply in the leaves can rather rapidly cause changes in the aperture of the stomata.

Material and Methods

The experiments were performed with wheat plants (Weibull's Eroica Wheat), cultivated in a nutrient solution containing $\text{Ca}(\text{NO}_3)_2$ 2×10^{-4} M, KNO_3 2×10^{-4} M, MgSO_4 10^{-4} M, KH_2PO_4 5×10^{-4} M and Fe citrate 10^{-5} M. The plants were cultivated in glass vessels under constant conditions (21°C , light from 100 W fluorescent lamps) and were about one week old when they were used for the experiments. For each experiment 6—8 plants were employed.

For the transpiration measurements the corona hygrometer was used. The method has been earlier described and discussed (1, 2, 22). Air of constant humidity is passed over the leaves which are enclosed in a cuvette, and after that conducted directly to the corona chamber. Here it passes a streamer corona discharge, which is dependent on the humidity of the air. In this manner the humidity can be measured and the output of water from the leaves can be continuously recorded.

During the experiments the leaves were enclosed in a cuvette and the roots hung down in a glass beaker with the nutrient solution. The beaker could be changed during the experiment and different substances could be added to the solution. It was found that a change of the solution to a new one with the same properties could not by itself affect the transpiration from the leaves. Air was constantly bubbled through the solution during the experiments with the twofold view of stirring and aerating the solution.

The changes of the properties of the root medium were undertaken after the plants had been illuminated during a period long enough for the stomata to open, and the transpiration to adopt a constant value. In wheat plants cultivated as described above, this is the case about 90—120 minutes after the start of the illumination.

Results

In the present experiments, the curves illustrating the changes in transpiration, are of three different types. An example of the first type is given in Figure 1. In this experiment the plants were standing in a nutrient solution with a total content of nutrients of 10^{-3} M. At the arrow an amount of a highly concentrated solution of the same composition was added. The addition was so adapted that the solution turned 10^{-2} M. A sudden increase was obtained, the start of which could be recorded within half a minute after the addition. Curves of the same type are obtained if the balanced solution is

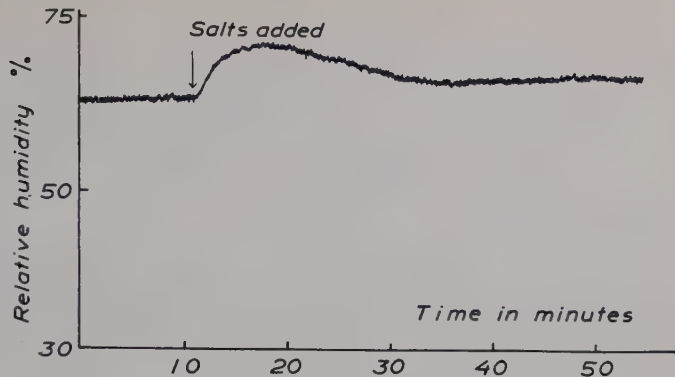


Figure 1. Continuous record of the transpiration of wheat leaves. At the arrow the quantity of nutrients in the root medium was increased from 10^{-3} M to 10^{-2} M.

changed to a one salt solution. Nitrates of the cations Li, Na, K, Rb, Cs, Ca, Ba, and Al have all been used. (In some additional experiments chlorides were used. Identical results were obtained.) The experiments were performed in two different ways. In one series the one salt solution was added to the basic medium with a total concentration of 10^{-3} M. The solution was made 10^{-2} M with respect to the added salt. In another series the basic medium 10^{-3} M was changed for a one salt solution 10^{-3} M. In both of the series the results were similar and a sudden increase of the transpiration was always recorded. An increase was also obtained as an immediate effect of a fall in the temperature in the root medium (Figure 2). The increase is transient and turns gradually to a decrease. The temperature fall was obtained by changing the beaker with the nutrient solution for another with an identically composed solution but with the new temperature. The opposite effect or a decrease in transpiration was found when temperature was raised. In both cases the result could be recorded within half a minute. Thus, rapidly entering

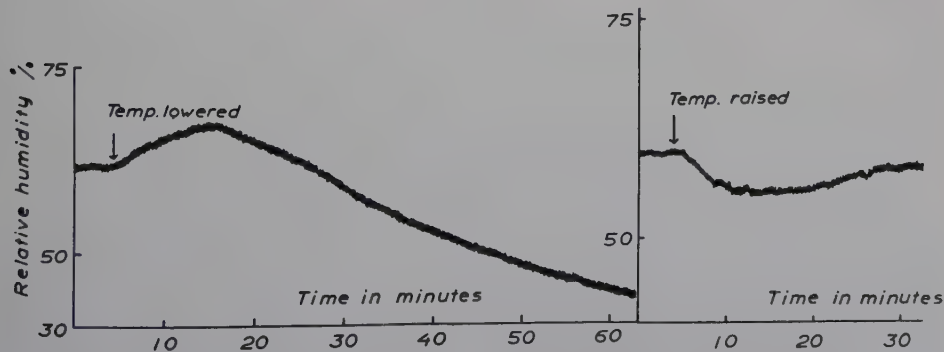


Figure 2. Effects of temperature changes in the root medium on the transpiration of wheat leaves. To the left $17^{\circ}\text{C} \rightarrow 2^{\circ}\text{C}$; to the right $9^{\circ}\text{C} \rightarrow 27^{\circ}\text{C}$.

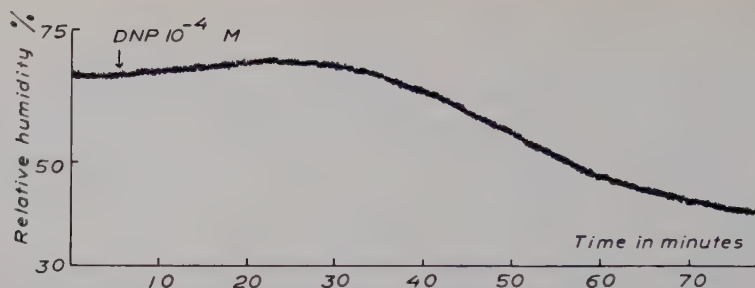


Figure 3. Effects of 2,4- dinitrophenol (DNP), added to the root medium, on the transpiration of wheat leaves.

changes of transpiration have been found to take place in both directions; an increase can be elicited by severing the leaf in air, by addition of different ions to the nutrient solution and by temperature fall, a decrease by severing a leaf under water and by an increase in temperature.

A second type of curves, obtained in the experiments is shown in Figure 3. The change in transpiration can be recorded firstly after some time (10—20 minutes). The length of the lag is dependent on the light intensity used (unpublished results of the author). Until now only decreases have been recorded. They have been obtained after the addition of different respiratory inhibitors to the solution. Figure 3 shows the result of an addition of 2,4-dinitrophenol (DNP). $\text{DNP } 5 \times 10^{-5} \text{ M}$ is active. This concentration increases the oxygen consumption of wheat roots (6). Similar effects on the transpiration are elicited by $\text{NaN}_3 \text{ } 10^{-4} \text{ M}$ and diethyl-dithio-carbamate DIECA (14). The decreases are reversible and if the inhibitor is removed by washing the roots with nutrient solution the transpiration increases again.

Finally the third type of curve (type III) which has been obtained in the experiments can be described as a combination of type I and type II. A sudden increase of the transpiration appears but it is soon changed to a decrease. The moment of the appearance of the decrease cannot be determined with any degree of accuracy and seems to be dependent on the light intensity used. This problem will be further illustrated elsewhere. Curves of type III are obtained after the addition of organic solutes as ethanol and diethyleter and of some heavy metals as Ag, Hg, and monoiodoacetic acid. Whether type III really is a combination of type I and type II cannot be settled at present but one example where this seems to be true will be described.

When the plants are placed in the basic medium with a total concentration of salts 10^{-3} M , the addition of DNP does not affect the transpiration at all. If a solution 10^{-2} M is used, however, the transpiration is decreased as described above (Figure 3). The curve is one of type II. An increase of

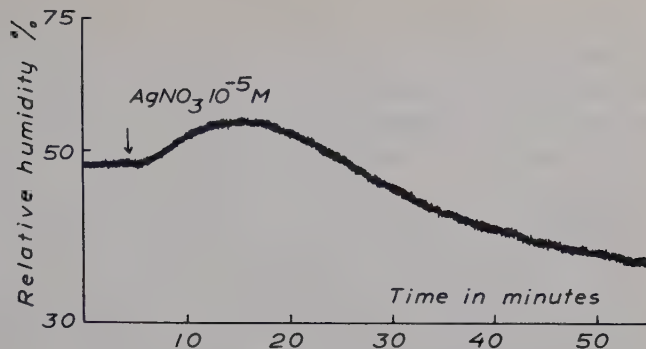


Figure 4. Effects of Ag ions, added to the root medium, on the transpiration of wheat leaves.

the concentration from 10^{-3} to 10^{-2} M gives an effect according to type I (Figure 1). Therefore the following experiment was performed. The plants were placed in a solution 10^{-3} M and DNP was added. No effect was obtained. 50 minutes later the concentration was raised to 10^{-2} M . The transpiration was immediately increased and rather soon changed to a decrease. The effect can be described by a type III curve. In this case it must be considered probable that the type III curve is a combination of one type I and one type II curve.

Discussion

There can be little doubt that the reason for all the types of changes in transpiration described in this paper must be looked for in the properties of the root system. The changes of type I take place so rapidly that no other part of the plant can be held responsible. In unpublished experiments the author has found that the transpiration of excised wheat leaves can be affected by agents added to the solution, in which they are standing, firstly within 10—15 minutes after the addition. Changes of type II, called forth by inhibitors, are reversible by washing the roots. However, it is possible to localize the effects to the roots only if an unbroken water column is present and easily movable within the whole plant body. Only then can a change in the root give an immediate response in the leaves. The presence of vessels in the vascular tissues and the theory of Strugger (18) that the water is preferably transported in the cell walls in parenchyma cells may justify a supposition of an unbroken and movable water column. A second condition that must be fulfilled is, that an excess or a deficit of water in the cell walls in the leaves, rapidly affects the water content of the guard cells of the stomata. The problem was discussed in the introduction and it was found that water uptake in the guard cells probably can take place rapidly.

Of course this does not imply that the water relations in the leaf cells, surrounding the guard cells has little importance for the width of the stomata, only that rapidly entering changes of the water stress in the cell walls may temporarily have great effects.

The following discussion is based on the assumption that the changes in the transpiration is caused by changes in the water influx in the roots. According to Theorell (20) the flux equation for transport of substances through a membrane can be written:

Rate of Transport = Concentration \times Mobility \times Sum of Driving Forces.

In the equation mobility or the reverse of resistance can also be changed to permeability, which is commonly used by plant physiologists. Concentration and Sum of Driving Forces will together form the suction from transpiration and the "root pressure". It has already been stated that in the present experiments the change of the transpiration must be caused by changes in the properties of the roots, *e.g.* the root pressure. Three possibilities as to the origin of the root pressure have been discussed in the literature; osmotically, actively (metabolically), and electrically induced water uptake. As to permeability rather little is really known of its details and it is by some authors linked to solubility, by others to physical activities. Thus we have several possibilities for reasons of changes in the water influx.

Both the rapid changes of type I and the slower changes of type II have been described earlier (14). Type I was then suggested to be caused by a change of the water permeability of the root and type II by an inhibition of the root pressure. In the present experiments nothing contradicts this interpretation but it is on the other hand not confirmed. The reason for the rapid type I changes would presumably be looked for in changes in physical properties. An electrically induced water uptake would be driven by a potential difference between the root surface and the water conducting system. Fensom (7) has found a connexion between changes in the rate of bleeding from excised roots and this potential difference. Studener (19) has measured the potential difference between the protoplasm and the surrounding medium of *Nitella* cells and found that a change of this potential, caused by the addition of different ions was established within 3—4 minutes. The earliest measurements were undertaken after half a minute. Further the Theorell-Meyer-Sievers theory for ion transports in ionic membranes gives points of agreement with the present results. Two reservations must be stated. Firstly that an electrically induced water flow takes place in the root and secondly that water transport can be involved in the theory.

The type II effects can be explained in different ways and may have different origins in the different experiments. Only the effects of respiratory inhibitors will be further mentioned. The results confirm earlier experiments

by Rosene (12, 13) Oota (11) and others. It is interesting to note the decrease of the transpiration in DNP solutions, which increase the oxygen uptake of wheat roots (6). This points to an uncoupling of the oxidative phosphorylation, which has been stressed by Oota. The fact that the fall in transpiration is obtained in a 10^{-2} *M* nutrient solution but not in a 10^{-3} *M* solution will be further investigated.

Döring (5), Nitsche (10) and others have investigated the transplantation reactions (Ueberführungsreaktionen), the changes in transpiration and water uptake, obtained after a sudden change of the temperature of the root medium. They have all found that transpiration as well as the water uptake decreases when temperature falls and increases when temperature is raised. However, none of these investigators could follow the progress of the phenomena continuously and were able to make observations only with some waste of time. Döring (5) has determined the temperature sensitivity of the water uptake of different species. Mostly he found a considerably decreased uptake when temperature was lowered from 20° to 0°C. However, a few species showed an increase and between the extremes a series of intermediates was found. In all cases investigated he found a decrease in the bleeding from excised root systems and therefore the root pressure always decreased with temperature. In an extensive discussion Döring concludes that his results can be explained only on the assumption that water permeability of the root in special cases is higher at low temperature than at high and that this factor to a great extent determines the uptake of water. This forms an interesting parallel to the present results.

Summary

Changes in transpiration, caused by changes of the properties of the root medium are recorded with a corona hygrometer. The changes can be divided into three groups.

1. Rapidly appearing changes, which take place within half a minute. Increased transpiration is elicited by an increase of the concentration of nutrients from 10^{-3} *M* to 10^{-2} *M*, by the addition of one salt solutions, and by a decrease of temperature. Decreased transpiration is called forth by an increase of temperature.

2. More slowly entering decreases of transpiration, appearing within 10—20 minutes. They are elicited par example by DNP and NaN_3 .

3. A rapidly entering increase of the transpiration, which is soon changed to a decrease. This type of curves is assumed to be a combination of the

foregoing curves. They are obtained by addition of alcohol, heavy metals as Ag and Cu, and monoiodoacetic acid.

The possible background of the transpiration changes is discussed. The changes must be due to changes in the properties of the root system. Earlier assumptions that the rapidly entering changes are caused by changes of the water permeability of the root and that the slower changes are caused by changes in the root pressure are not contradicted.

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The Influence of Growth Conditions on the Amount and Ribonucleic Acid Content of Wheat Root Mitochondria

By

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By experiments on wheat roots Burström *et al.* (1952, 1954, 1955) have shown that the roots are greatly influenced by variations in the concentration of calcium and auxins in the nutrient solution. The relations between the amount of growth-regulating substances and the amount of mitochondria in the root cells have been studied by Florell (1956, 1957). He found that a decrease in the calcium concentration of the nutrient caused a decrease in the amount of mitochondria, whereas treatment with growth-promoting substances, such as phenylboric acid (PhBA) and α -*para*-chlorophenoxy-*iso*-butyric acid (PCIB), had no effect on the amount of mitochondria.

The chemical composition of the mitochondria has been examined by Stafford on pea seedlings and by Florell (1956) on wheat roots. According to these analyses the mitochondria consist of 30—40 per cent proteins. Stafford has also shown that the lipids amount to 30 per cent and that the content of nucleic acids does not exceed 2 per cent.

The present investigation is an attempt to find out whether the size of the mitochondrial fraction (which is here called the amount of mitochondria) in wheat roots is influenced by 1-naphtyl-acetic acid (NAA), which inhibits the root growth, and to determine the amount of mitochondria in wheat roots cultivated in distilled water in order to see whether the absence of calcium has the same effect as a decrease in the calcium concentration of the nutrient.

Florell (1956) has shown that the proportion of protein does not change with varying calcium concentration. Since the amount of the ribonucleic acid of the mitochondria is of importance to their function, it will also be examined

whether there are any changes in the amount of ribonucleic acid when the roots are cultivated in nutrient with and without addition of growth-regulating substances and after cultivation in distilled water.

I. Methods

The test material was young plants of Weibull's Eroica Wheat. The seeds were germinated for 48 hours on moist filter paper at 22°C and then transferred to 1-litre-beakers containing 800 ml. of the nutrient solution with the following composition: KNO_3 $5 \cdot 10^{-3}$ M, KH_2PO_4 $5 \cdot 10^{-3}$ M, Na_2HPO_4 $5 \cdot 10^{-3}$ M, MgSO_4 $2 \cdot 10^{-3}$ M, Fe-citrate $2 \cdot 10^{-5}$ M, MnSO_4 10^{-5} M, CaCl_2 10^{-4} and 10^{-7} M. PhBA was used in the concentration 10^{-5} M and NAA in 10^{-6} M. Plants were also cultivated in distilled water, which as in the other experiments was free from heavy metals.

The plants were grown under artificial illumination at 23°C. Air was bubbled through the solutions, which were changed every other day. The cultivation period was 4 days where nothing else is mentioned. The pH was 6.9 in all series except in distilled water where it was 5.3.

All series comprised the roots of 250 plants, from which 200 were used for the preparation of the mitochondrial fraction. Dry weights were determined after drying at 105°C for 48 hours. The primary root has been measured on each plant and the mean is given as the root length.

The mitochondrial fraction was prepared according to the method of Withrow and Wolff. Trietanolamine was used as a buffer. The pH was 7.1. About one third of the mitochondrial fraction was used for dry weight determination and the residue for extraction of nucleic acid as described by Ogur and Rosen. In the nutrient solution series it proved impossible to extract all RNA with cold perchloric acid. The extract contained only a small and in different experiments varying amount of RNA together with an unknown material with a strong ultraviolet absorption. It proved impossible to eliminate this material in spite of several experiments with varying extraction methods. The extraction was continued in hot perchloric acid, and an extract was obtained whose absorption curve showed a maximum at 2600 Å.

The temperature was kept at 2°–4°C from the harvest up to the extraction in cold perchloric acid in order to reduce the enzymatic activity as much as possible.

Since RNA had been extracted in perchloric acid and from plant material, and since RNA was estimated by the method of Ogur and Rosen, $\epsilon(\text{P})_{2600}$ was assumed to be 10,800. The same assumption has been made by Bhargava, Simkin and Work.

Table 1. Dry weight of the mitochondria in per cent of the dry weight of the roots after homogenizing with and without EDTA.

EDTA M	+ EDTA	— EDTA
$5 \cdot 10^{-4}$	3.3	2.9
$1 \cdot 10^{-3}$	3.5	3.4
$5 \cdot 10^{-3}$	3.0	3.2
$1 \cdot 10^{-2}$	3.5	3.5

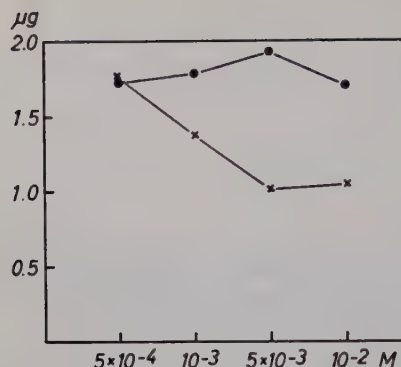


Figure 1. The amount of RNA-P found in the hot HClO_4 -extract after homogenizing with different versene concentrations in the sucrose-buffer solution. On the abscissa the concentration of versene in M; on the ordinate μg . RNA P/mg. dry weight of the mitochondria. Circles — without versene, crosses — with versene.

A Beckman Spectrophotometer model DU was used for measurement of the optical density. The phosphorus estimations were made according to the method of Allen with certain modifications (Bergkvist). A Klett-Summerson photocolormeter was used. Experiments were made to determine RNA by ribose determination with orcinol, but this method gave values which highly exceeded the results of the above-mentioned analysis. The method was considered unsuitable on this material. Martin and Morton also showed with material from Beta that quantitative determination of RNA with orcinol on plant material is not feasible.

Determination of deoxyribose with diphenylamine proved no deoxyribonucleic acid (DNA) to be present. Only exceptionally, probably when nuclei were destroyed, small mounts of DNA could be found. Levitt and Millikan have not been able to show any DNA in the mitochondria from potatoes, whereas Stafford reports 0.7—0.9 per cent DNA in the mitochondrial fraction from pea seedlings. According to Hogeboom and Schneider DNA does not normally occur in the cytoplasm.

Lund, Vatter and Hanson have supposed that when preparing the mitochondrial fraction without presence of versene non-mitochondrial material might sediment, in which case the weight of the fraction would be incorrect.

Preliminary experiments were made with varying concentrations of versene being added to the sucrose-buffer solution in order to see whether it was possible to obtain a purer mitochondrial fraction. Table 1 shows that the amount of mitochondria does not show any changes caused by addition of versene.

On the other hand, the experiments show that versene concentrations greater than 10^{-3} M cause a decrease in the content of the RNA of the mitochondria dissolved in hot perchloric acid. (In the cold perchloric acid there was no change in the content of RNA.) Even at 10^{-3} M versene it seems doubtful whether versene should be used (Figure 1).

Tedeschi and Harris have shown that the mitochondria possess a semi-permeable membrane similar to the tonoplast; as we know that calcium regulates the permeability of the plasm membranes, it may have the same effect on the membrane of the mitochondrion (Florell 1956). The reduction of the calcium concentration caused by the addition of EDTA thus would involve a change in the permeability of the membrane of the mitochondrion. For this reason a large part of the RNA, which is soluble in hot perchloric acid, would be lost at an early stage.

II. Experiments and Results

1. Distilled Water

In order to find out the importance of the nutriment in the endosperm for the root-growth and for the mitochondria and their content of RNA, plants were cultivated in distilled water and controls in nutrient solution. The growth in length of the root and the formation of dry substance and the absorption of water occur in the beginning about as quickly in distilled water as in nutrient solution. Later on the difference will be greater.

It is remarkable that the absence of salts in the cultivation medium is followed by changes in the results of the RNA analyses. The total amount of RNA P is the same in cultivation in distilled water and in nutrient solution but the solubility of the RNA is essentially reversed.

When the roots are cultivated in distilled water, the absorption curve of the cold perchloric acid fraction shows a maximum at 2600 Å and the absorption curve of the hot fraction is flatter (Figure 2). When the plants are cultivated in nutrient solution it is just the contrary. This will be discussed later on.

2. Calcium

Wheat plants were cultivated in nutrient solution at two different calcium levels, 10^{-4} and 10^{-7} M. Ca 10^{-4} M gave maximum values of root length, fresh weight and the dry weight of the mitochondria (Florell 1957). Ca 10^{-7} M was chosen because total absence of calcium gives a rather irregular root growth.

Table 2 shows that an increase in the calcium concentration from 10^{-7} M to 10^{-4} M involves an increase in the amount of mitochondria of about 50 per cent which confirms earlier investigations.

Figure 2. Absorption curves of extracts in cold (—) and hot (---) HClO_4 after cultivation in dist. water (A) and nutrient solution (B). On the abscissa the wave-length (Å); on the ordinate the optical density.

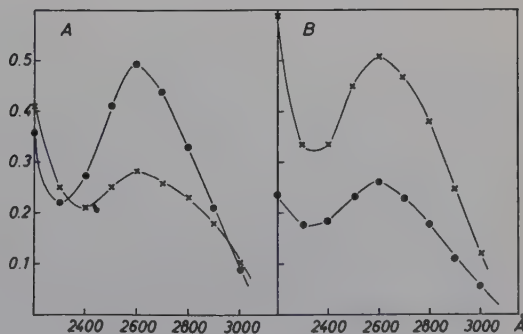


Table 2. *Root length per plant, fresh weight, dry weight, and dry weight of the mitochondria of 200 plants cultivated in dist. water only and in nutrient solution with different calcium concentrations and after addition of growth regulators. The variation between the controls (Ca 10^{-4}) is due to variations in the material during the long time over which the experiments were extended.*

Treatment	Root length mm	Fresh weight g	Dry weight g	Dry weight of mitochondria mg
Dist. water	68.6 \pm 5.6	10.30 \pm 0.50	0.86 \pm 0.03	17.5 \pm 0.9
Ca 10 ⁻⁷	47.6 \pm 3.3	7.02 \pm 0.47	0.62 \pm 0.04	21.9 \pm 1.8
10 ⁻⁴	97.0 \pm 2.7	14.73 \pm 0.52	0.96 \pm 0.04	32.9 \pm 1.6
PhBA 10 ⁻⁵	154.1 \pm 2.6	18.42 \pm 0.38	1.07 \pm 0.03	28.2 \pm 1.6
0	103.4 \pm 1.9	15.67 \pm 0.37	1.06 \pm 0.03	30.9 \pm 2.0
NAA 10 ⁻⁶	42.4 \pm 0.8	9.05 \pm 0.19	0.81 \pm 0.02	23.4 \pm 1.0
0	98.5 \pm 2.0	14.09 \pm 0.40	1.00 \pm 0.04	27.0 \pm 1.4

It is seen from Table 3 that the content of RNA P of the mitochondrial fraction is not significantly affected by variations in the calcium concentration.

3. Phenyl Boric Acid and Naphthylacetic Acid

Experiments were made with PhBA and 1-NAA in order to examine whether various growth-regulating substances influence the amount of mitochondria and their RNA content.

Addition of PhBA does not seem to involve any significant change in the amount of mitochondria (Table 2) and their content of RNA P is not influenced either (Table 3).

Table 2 shows that addition of NAA to the nutrient solution has no obvious effect on the amount of mitochondria and their RNA P content (Table 3).

Table 3. *RNA-P μ g. per mg. dry weight of the mitochondria. Cultivation as described in Table 2.*

Treatment	UV-analysis			P-analysis		
	Cold HClO ₄	Hot HClO ₄	Cold + Hot	Cold HClO ₄	Hot HClO ₄	Cold + Hot
Dist. water	1.50 \pm 0.15	0.60 \pm 0.04	2.10 \pm 0.17	1.56 \pm 0.18	0.74 \pm 0.06	2.30 \pm 0.23
Ca 10 ⁻⁷	0.60 \pm 0.06	1.61 \pm 0.07	2.21 \pm 0.09	0.34 \pm 0.12	1.60 \pm 0.09	1.94 \pm 0.12
10 ⁻⁴	0.62 \pm 0.07	1.62 \pm 0.07	2.23 \pm 0.09	0.36 \pm 0.08	1.50 \pm 0.08	1.86 \pm 0.06
PhBA 10 ⁻⁵	1.22 \pm 0.06	1.55 \pm 0.10	2.77 \pm 0.13	0.84 \pm 0.13	1.50 \pm 0.06	2.34 \pm 0.13
0	1.15 \pm 0.05	1.63 \pm 0.08	2.78 \pm 0.10	0.80 \pm 0.08	1.49 \pm 0.10	2.29 \pm 0.09
NAA 10 ⁻⁶	1.05 \pm 0.05	1.56 \pm 0.12	2.60 \pm 0.14	0.59 \pm 0.10	1.37 \pm 0.07	1.96 \pm 0.10
0	0.98 \pm 0.11	1.44 \pm 0.10	2.42 \pm 0.17	0.54 \pm 0.05	1.38 \pm 0.09	1.92 \pm 0.09

III. Discussion

1. *Growth and Amount of Mitochondria*

The normal growth of the plants cultivated in distilled water depends on the comparatively rich supply of organic and inorganic nutriment in the endosperm, which is sufficient for experiments of short duration. Bosemark, for instance, has shown that wheat plants can nourish on the endosperm for four days and that the supply of nitrogen is exhausted only after seven days.

On cultivation in nutrient solution with calcium 10^{-4} M and 10^{-7} M there were greater differences than between roots cultivated in distilled water and in nutrient solution at the calcium level 10^{-4} M. This is true of appearance as well as growth.

It must be considered as established that the amount of mitochondria is changed through the influence of calcium. The possibility remains that the low amount of mitochondria discovered is not due to a smaller formation of mitochondria in the cells but to the fact that their duration is dependent on the calcium concentration. A lower content of calcium would mean that the mitochondria only for a short time had such a condition as to be present in the mitochondrial fraction. The question will be discussed later on.

The results with PhBA and NAA show that these substances do not change the amount of mitochondria in the wheat roots.

PhBA, a substance investigated by Torsell, increases the cell elongation powerfully, but its mode of action is unknown and there are no structural reasons for including it among the auxins (Burström 1957).

Thus PhBA, NAA and as earlier mentioned PCIB have a great effect on the root growth but do not affect the amount of mitochondria or their content of RNA, and Eliasson has shown that the respiration is not affected either. This indicates that the growth cannot depend on greater variations in the energy- or the basal metabolism. Nor does the variations of the extension growth cause disturbances of the cytoplasmic organization.

2. *The Ontogeny of the Mitochondria*

To investigate the increase in the amount of mitochondria during the root growth, determinations were made on three-day-old plants and these values were compared with the values from four-day-old plants.

It appeared that the amount of mitochondria did not increase significantly during this time. The mean for three days (five experiments) was 31.4 mg. per 200 plants while the mean for four days was 32.9 mg.

Since there is a continuous formation of mitochondria in the root cells, this very low increase is surprising.

The most plausible explanation seems to be the following. The mitochondria formed in the roots during the evolution of the cells cannot be found in the mitochondrial fraction on account of their decrease in weight or their decomposition. Of these alternatives the latter seems to be the more probable, even if mitochondria have been found in animal embryonic material, which are more or less empty (Weissenfels 1958) and consequently have decreased in weight, a fact which eventually would support the former theory.

Different results have been published concerning the cytoplasmic particles, their origin, their size and activity and the connection between them (cf. Chantrenne 1947, Green 1952, Elson and Chargaff 1952, Smellie *et al.* 1953, Kmetec and Newcomb 1956, Müller 1956). Even if certain investigations indicate (Claude 1938, 1943 and Kuff and co-workers 1956) that the different particles can be classified into restricted groups of size and even if there is no generally accepted view as to their origin, there is much indicating that at least certain of the particles can be derived from each other.

The cause of the hypothetic decomposition is unknown, but one might consider a spontaneous decomposition regulated by endogenous factors (for instance the ration surface area: volume). Our knowledge of the ageing of the cells and cell particles is very limited, however, and hardly lends support to a discussion. For this reason an attempt will be made to connect the decomposition with changes in external factors such as osmotic variations or changes in the concentration or balance of ions of the cell. Farrant and co-workers have found that the structure of the mitochondria from *Beta vulgaris* is much more labile than the structure of the animal mitochondria. Taft and Levitt have confirmed this and have shown that the mitochondria promptly expand in hypotonic and contract in hypertonic solutions. The concentration of the cell sap is generally lower in the zones where the cells are intensely elongating.

Burström (1942) has shown, however, that the osmotic value in the elongation zone of the root remains constant if the nutrient conditions are optimal. In the different parts of the cell there might still be small variations in the concentration of ions and during the transport in the cell, specially when the cell is beginning to elongate, the mitochondria might be exposed to variations in the osmotic conditions, which might involve lessened stability of the structure.

But it is perhaps most plausible to connect the decomposition of the mitochondria with the absorption of ions.

It is rather generally supposed that the absorption of ions is mediated by carriers, at least under certain circumstances. Different carriers have been proposed, among others Robertson, Wilkins, Hope and Nesztel have proved that the mitochondria accumulate salts just as the cells do, and Bartley and

Davies have shown that mitochondria accumulate more ions from the medium than what corresponds to the equilibrium of concentration. Lansing, Rosenthal and Tanada consider the role of RNA in the absorption of ions. The mitochondria are most often mentioned among these hypothetic carriers.

It seems well grounded to examine the mitochondria or their RNA as carriers of calcium, specially since it has been shown that this ion influences the amount of mitochondria in wheat roots.

If the mitochondrial substance during (Russel 1954) or after the formation of organized mitochondria absorbs calcium or if the mitochondria formed through self-reproduction absorbs this ion, then the influence of calcium on the colloid substance would prevail and the structure of the mitochondria would change. Afterwards, when the mitochondria with their more or less loosely bound calcium are carried about in the cell either actively through their self-motility or passively through plasma streamings, they are exposed to an ionic equilibrium which is changed during the cell elongation. Then the calcium balance of the mitochondria will be disturbed and calcium ions will be freed. The stabilizing effect of calcium on the mitochondria will come to an end and the mitochondria will break down.

During their ontogeny the mitochondria may give rise to particles of different size, morphologic structure and biochemical functions. During the first phase small particles may be formed from the mitochondrial substance or eventually through self-reproduction. These small particles may give rise to promitochondria and via these mitochondria may be formed more or less directly. During a second phase the mitochondria may serve as carriers and contain the enzymatic systems for the energetic processes. During a third stage the mitochondria may break down and eventually form particles with different functions, as the particles derive from different parts of the mitochondria, for instance, from osmiophil and non-osmiophil lamellae where some particles may have functions within the protein metabolism (*i.e.*, they appear as microsomes, if these are no characteristic particles but products of decomposition of the mitochondria or their early stage). At last the mitochondrial substance may to some extent form part of the tonoplast, which in Butler's opinion on account of functional similarity may have a similar composition to that of the mitochondria.

Certain facts supporting the supposition that the mitochondria decompose rather early have been presented in connection with the study of the localization of the cytochrome oxidase in the plant cell. The enzyme has been found in the mitochondrial fraction from plant cells younger than three days, but it is absent from particulate fractions of older cells (Waygood 1950). The enzyme is found in intact old cells according to spectrophotometric investigations by Lundegårdh (1952). In Butler's opinion the cytochrome oxidase here

is localized to the tonoplast. The mitochondria may have a rather short duration of life; when they decompose the cytochrome oxidase enters the tonoplast, which in the preparations of the particulate fractions will be so destroyed as to inactivate the cytochrome oxidase.

The decomposition of the mitochondria might be compared to the dissolution of the nuclear membrane and the nucleols during the mitose and the conversion of the chromosomes into chromatin. At this time the DNA-proteins and macromolecules of the chromosomes, which have been held together by calcium and magnesium, will separate when the ionic concentration of the medium goes below a certain value (Mazia).

3. *The Nucleic Acid Content of the Mitochondria*

When the nucleic acid content of the mitochondria is determined, it becomes evident that the solubility of RNA in cold or hot perchloric acid is wholly changed if the cultivation is made in distilled water or in nutrient solution.

According to Ogur and Rosen RNA can be dissolved in cold 1-N perchloric acid. Several workers (for instance Chiba and Sugahara 1957) have met with difficulties when extracting RNA in cold perchloric acid. The explanation of the varying reports might be the different cultivation substrate. Ogur and Rosen and Stafford have used only distilled water in their experiments with seedlings, while otherwise nutrient solution has been used or material has been taken from the green-house.

The presence of ions in the cultivation medium decreases the solubility of RNA in cold perchloric acid as has been shown here. Only 30 per cent of the RNA comes in the cold fraction while 70 per cent appears here if the material is cultivated in distilled water.

It should be mentioned that Martin and Morton have found two types of RNA in mitochondria from Beta: one that is soluble in cold 1-N perchloric acid and another which is dissolved in 10 per cent NaCl at 100°C.

This might be tentatively explained as follows. As carriers of ions and here especially the calcium ions the mitochondria might be more resistant against the cold perchloric acid when the calcium concentration is high. Only a treatment with hot perchloric acid would make the RNA wholly soluble. It is also possible that the RNA itself is affected by ions and changes its solubility.

It is rather uncertain, however, whether the calcium ion alone is the cause of the decreased solubility. In any case, the solubility is not better when there is a low calcium concentration in the nutrient solution. Many experiments were made in order to determine whether any other ion might be the cause of the changed solubility. Ca, Mg, Mn, K and nitrate ions were added to

distilled water, but the results were wholly negative. A successive removal of one ion at a time from a nutrient solution would perhaps give some suggestions.

There are rather few investigations concerning nucleic acids in plants. This is specially true of higher plants. The distribution of nucleic acid in different parts of the plant has been studied by Euler and Hahn, who have showed that the content of RNA-P in the rye plant decreases from the root to the shoot apex and Potapov and Maroti, who believe that nucleic acid is transferred to the shoot from the root where it is mainly synthesized. With roots from *Pisum Sudi* and Maroti have found a 50 per cent decrease in the content of RNA per cell during the cell elongation associated with a fourfold increase in its DNA-content.

A similar investigation has been made by Jensen who has concentrated on the first two and three millimeters of the root tips of *Allium cepa* and *Vicia faba*. He shows that the value of RNA, DNA and protein per cell of the meristem is the lowest found in the root. The DNA values per cell doubles as the divisions occur more frequently and the RNA values at first increase three-fold and then there is a leveling of the RNA content as the elongation begins.

Leslie has given a thorough account of the investigations concerning the problem of whether the content of nucleic acid can be influenced by the composition of the nutriment. It has been possible in animals to demonstrate variations in the content of nucleic acid with changes in the protein supply.

Silberger and Skoog have tried to influence the content of RNA by adding indoleacetic acid. With material from *Nicotiana* they have showed that high concentration of indoleacetic acid cause an increase in the total amount of RNA. At lower concentrations the content of DNA is increased.

Summary

The influence of calcium and growth regulators on the amount of mitochondria and their content of ribonucleic acid has been investigated. Small amounts of calcium give an increase in the mitochondrial fraction while the growth regulators have no influence on the formation of mitochondria. The ribonucleic acid content is not affected by variations of the calcium concentration or by growth regulators.

Differences in the solubility of the ribonucleic acid after cultivation in nutrient and in distilled water were observed.

The ontogeny of the mitochondria is discussed on the basis of an established relative decrease in the amount of mitochondria of older roots.

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Further Studies in the Physiological and Genetical Interrelations between Pyridoxamine- and Pyridoxal-deficient Mutants of *Ophiostoma*

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In a previous paper (Wikberg 1959) the requirements of two pyridoxamine-deficient strains of *Ophiostoma* were reported. The mutant strains were produced by ultraviolet light from the pyridoxine-deficient wild type, which grows equally well on pyridoxine, pyridoxal and pyridoxamine. From one of the mutants a pyridoxal-deficient strain which does not respond to pyridoxine was obtained. In the present investigation the physiological and genetical interrelationship between the strains was studied.

Materials and Methods

All the crosses were made in slanted agar culture tubes. As far as possible inocula for these crosses were taken from two- or three-day-old monoconidial mycelia of the strains being studied. The medium used was washed agar supplied with 0.2 % malt extract (Vitrum). After about a week mature perithecia had developed and ascospores were obtained. 1 ml. portions of appropriate dilutions of the spores were pipetted onto the agar surface of Petri dishes containing 10—15 ml. minimal medium (Fries' medium 3) supplied with pyridoxamine, 10 μ mol per plate. The dishes with the lids slightly open, were placed in a dry thermostat chamber at 30°C. After 24—36 hours the water of the spore suspension had evaporated and 1-ascospore mycelia could be isolated under the microscope and transferred to tubes with complete medium.

The identification of the progeny was made either in 30 ml. bottles or in shaken tubes with totally 10 ml. minimal medium with appropriate supplements. The quan-

titative responses of the B₆ vitamins were studied in shaken tubes according to the method devised by Fries (1949). In some cases 100 ml. flasks with 20 ml. minimal medium were used instead. The mycelia were collected, washed, dried at +105°C and weighed in glass crucibles.

The strains used were kept alive by re-inoculations every half year on complete agar medium. The following strains were used: W 233-2 (+), pyridoxamine-less, W 366-10 (+), pyridoxamine-less, 1 (—), wild type and 2 (+), wild type.

Data Regarding the Interrelationship between the Pyridoxamine-less Strains

The effect of pyridoxal. As described previously (Wikberg 1959) the quantitative response of both the pyridoxamine-less mutants, W 366-10 and W 233-2, to pyridoxamine is the same as that of the wild type to this vitamin. In contrast to the wild type the mutants do not grow on pyridoxine. Pyridoxal in large doses, however, produces growth after a lag of several days. Special attention was paid to the effect of pyridoxal on these strains, since one pyridoxal-less isolate, W 366-10-1, had been obtained from a culture of one of the pyridoxamine-less strains, W 366-10. This pyridoxal-less isolate was able to grow with pyridoxal in a concentration as low as that of pyridoxamine.

Mating experiments between the wild type and the mutants were made. The progeny was identified and the mutant strains in f₁ were recrossed to (+) and (—) strains of the wild type. The results are shown in Table 1. As seen both mating types are found among the mutants. The results indicate that the gene(s) responsible for the mutant character is probably not linked to the mating type locus. In order to ascertain if the slow response to pyridoxal in the pyridoxamine-less strains was strictly heritable, five of the substrains from the intercross of W 366-10 to wild type were analysed regarding their reaction towards pyridoxal. The strains were tested in shaken tubes and the tubes were supplied with the following concentrations of pyridoxal: 1, 3, and

Table 1. *The result of crosses between W 366—10 (+), pyridoxamine-less, and wild type and W 233—2 (+), pyridoxamine-less and wild type.*

Strains crossed	Number of 1-ascospore mycelia isolated in f ₁			
	Total	Wild type + or —	Pyridoxamine-less	
			+	—
W 366-10 × wild	51	31	13	7
W 233-2 × wild	64	41	11 ¹	4 ¹

¹ Mating type unknown for 8 strains (perithecia not formed).

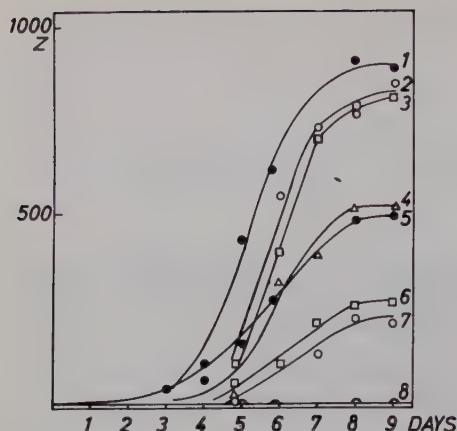


Figure 1. Growth (extinction values, Z) of W 366-10, pyridoxamine-less (\bullet), and three f_1 strains of this mutant, viz. W 366-10-12 (Δ), W 366-10-14 (\square), and W 366-10-15 (\circ), in shaken tube cultures supplied with pyridoxal 10 μmol (curves 1, 2, 3, 4), 3 μmol (curves 5, 6, 7) and 0 μmol per tube (curve 8).

10 μmol per tube. The growth curves for three of the f_1 strains are shown in Figure 1. Corresponding curves for the original mutation W 366-10 are also given. Variations in the growth response among the different strains are noted. The growth lag is about the same in all cases, but two of the substrains respond to 3 μmol of the vitamin, whereas the third does not grow at all with this amount of pyridoxal. No growth at all was obtained with 1 μmol pyridoxal. The final extinction values for the same amount of vitamin varied considerably for the isolates. Presumably these variations have their origin in the influence of the genetic background, which is probably different for the different substrains. The variations reported may, of course, also be ascribed to other causes, *e.g.* differences in the physiological conditions of the inocula of the strains. However, since the same main differences were noted also when the experiment was repeated it may be assumed that an interference of other genes in the phenotypic expression of the mutation really occurs in this case. Such effects have been reported from histidine-less strains of *Neurospora*, which were isolated from the same ascus after a cross between the histidine-less mutants and the wild type (Mathieson and Catchside 1955).

The progeny of the other strain, W 233-2, when crossed to wild type was not investigated so thoroughly as to its response to pyridoxal, but qualitative observations indicate that on the whole the f_1 strains do not deviate from the mother mutant or from W 366-10 in this respect. With variations within certain limits the response pattern for pyridoxal is therefore heritable in both mutant strains.

In the search for physiological differences between the two pyridoxamine-deficient strains cross-feeding experiments were also performed in a pyrid-

Table 2. *The effect of pyridoxine, pyridoxal and pyridoxamine on the growth of pyridoxamine-deficient W 366-10 at 20°C. Dry weights of mycelium in mg. after 6 days.*

Vitamin added	μmol vitamin per flask		
	1	10	100
Pyridoxine	6.9	11.3	12.5
Pyridoxal	5.3	11.3	22.3
Pyridoxamine	51.0	53.6	55.8

oxine-supplemented medium. Qualitative tests with mixed suspensions of conidia from the mutants in flasks were negative. One experiment was also performed on a more quantitative basis with W 233-2 and the pyridoxal-less W 366-10-1. Varying concentrations of the inoculation material (*viz.* $0.2 \cdot 10^6$, $1 \cdot 10^6$ and $25 \cdot 10^6$ conidia per tube of each strain) were combined with 1, 3, and 10 μmol pyridoxine per tube. No growth was obtained, however. The same test for the combination of W 366-10-1 and W 366-10 has not yet been made.

The effect of temperature. One difference in growth behaviour has been found between the mutants, *viz.* the reaction towards pyridoxal and pyridoxine at 20°C. Table 2 records data from an experiment in which W 366-10 was tested for its response to these vitamins at 20°C in 100 ml. flasks with 20 ml. minimal medium per flask. For comparison a series with pyridoxamine was included. The concentrations used were 1, 10, and 100 μmol vitamin per flask. Each flask was inoculated with about one million conidia and incubated for 6 days. The dry weights in the table are the mean of the weights of mycelium obtained from two flasks. In control series no visible growth was obtained. The positive effects of pyridoxine and pyridoxal are evident for W 366-10. At this temperature the block for these vitamins is relieved, but only partially since both are inferior in activity to pyridoxamine. Strain W 366-10 thus represents a "temperature-sensitive" mutant in the sense of Beadle and Tatum (1945). The other strain, W 233-2, and nine of its sub-strains in f_1 did not grow at all when tested under these conditions.

Genetic data regarding the interrelationship between the mutants. An inter-cross has been made between the two pyridoxamine-deficient mutants, using W 366-10 (+) and W 233-2-3 (—), the latter derived from a cross between W 233-2 (+) and the wild type. From one plating out of ascospores 140 mycelia were isolated. 63 of these were temperature-sensitive when tested on minimal agar plates supplied with pyridoxine. Since they formed mycelia at 20°C, they were supposed to be of type W 366-10. The remaining mycelia did not grow at 20°C and thus belonged to type W 233-2. Of these 140 only one mycelium was prototrophic. This single mycelium cannot, of course, be taken

as a proof of recombination, since it might have been formed by a reversion to prototrophy of a mutant ascospore or conidium at some stage of the mating experiment.

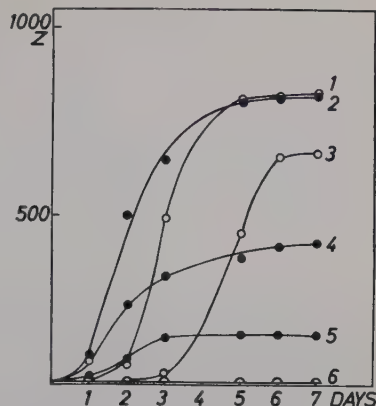
A higher number of prototrophic mycelia could, however, be obtained when a suspension of ascospores from a cross between the mutants was plated out on minimal agar medium supplemented with pyridoxine. Since the ascospores have a tendency to stick together, forming aggregates, it was not possible to determine the proportions between mutant and prototrophic spores. This experiment together with the one mentioned above permits the conclusion that the two pyridoxamine-deficient mutants are non-allelic and that the two genes in question are closely linked. Another possibility is that they are pseudo-alleles of the same gene.

Experiments with the Pyridoxal-deficient Strain

The effect of pyridoxal and pyridoxine. The pyridoxal-deficient isolate W 366-10-1, which has been described previously (Wikberg 1959), cannot utilize pyridoxine, at least when this vitamin is tested alone. In order to investigate whether pyridoxine had any effect together with pyridoxal both vitamins were tested in an experiment in shaken tubes, using the following combination: pyridoxal 0.10 μmol +pyridoxine 0.10, 0.30, 1.0, 3.0, and 10.0 μmol per 10 ml. medium. The results for three combinations are shown in Figure 2. Pyridoxine inhibits growth of the pyridoxal-less strain by competing with pyridoxal. At a pyridoxine-pyridoxal ratio of about 30:1, growth is not initiated for several days. At lower ratios the inhibitory effect of the vitamin manifests itself by a growth lag of variable length. In no case did the final growth values exceed that obtained with pyridoxal alone. This fact indicates that pyridoxine is not utilized at all by the pyridoxal-less mutant W 366-10-1, and thus there is probably a total block after pyridoxine in this strain.

The heritability of the character pyridoxal-less. The pyridoxal-less strain W 366-10-1 had been isolated from a culture of the pyridoxamine-less mutant W 366-10 grown on a medium supplied with pyridoxal. Therefore the strain may be looked upon as being formed from the pyridoxamine-deficient one by "adaptation" to the substrate used for the conidial suspension from which it was isolated. There are two possible explanations for this "adaptation". One is that the pyridoxal-less strain is a substrate-activated modification of the pyridoxamine-less one, which means that the phenomenon must be classified as "enzymatic adaptation" (Stainer 1951) and the change in phenotypic behaviour need not necessarily involve a change of the genotype. Removal of

Figure 2. Growth (extinction values, Z) of W 366-10-1, pyridoxal-less, in shaken tube cultures with different additions. a) Pyridoxal 0.10 μmol (curve 2), 0.03 μmol (curve 4), and 0.01 μmol per tube (curve 5). b) Pyridoxal 0.10 μmol +pyridoxine 0.3 μmol (curve 1), pyridoxal 0.1 μmol +pyridoxine 1 μmol (curve 3) and pyridoxal 0.10 μmol +pyridoxine 3.0 μmol per tube (curve 6).



the activating substrate, in this case pyridoxal, will then result in deadaptation. The second possibility is that the pyridoxal-less strain has been formed through mutation from the pyridoxamine-less one.

Substrate-activated adaptation was not excluded by the experiments described previously, since the presumed activating substrate pyridoxal had been used as supplement to the minimal medium when the inoculum for the growth experiments was prepared.

In order to study the stability of the pyridoxal-less strain 3 serial transfers were made on medium supplied with pyridoxamine. These were followed by a growth experiment in shaken tubes, the inoculum for which was obtained from a conidial suspension of the last transfer. The concentrations of pyridoxal were 0.03 and 0.10 μmol per tube. Reference series with pyridoxamine and pyridoxine were included. The result was the same as that obtained when pyridoxal was used as a substrate for the inoculum, leading to the conclusion that no deadaptation had taken place, and that "enzymatic adaptation" was not the cause of the deviating character of W 366-10-1.

Thus the modification was stable, and if it was due to mutation, which was likely, a population of conidia of W 366-10 grown on pyridoxamine medium probably contained a certain frequency of pyridoxal-less conidia. Pyridoxal-less mycelia from such a population were screened in the following way. Serial dilutions of a washed two-day-old suspension of conidia from W 366-10, which had been produced on a medium supplied with pyridoxamine, were plated out in minimal agar medium containing 0.3 μmol pyridoxal per plate. The densities used were $2 \cdot 10^7$, $2 \cdot 10^6$, $2 \cdot 10^5$, $2 \cdot 10^4$, and $2 \cdot 10^3$ conidia per plate, 2 plates of each. After incubation for 3 days at 30°C 50 mycelia had developed on these plates. Conidia from these mycelia were transferred to minimal medium containing 10 μmol pyridoxine per plate.

Table 3. *The result of a cross between W 366-10-1 (+), pyridoxal-less, and wild type 1 (—).*

Total	Wild type	Number of 1-ascospore mycelia isolated in f_1			
		Pyridoxal-less		Pyridoxamine-less	
		+	—	+	—
66	42	5	5	9	5

Out of these 50 transfers 4 did not start growing. These were isolated on complete agar medium, and one of them was tested further for its quantitative response to pyridoxal. The result was the same as that for the previously isolated pyridoxal-less strain, W 366-10-1. — The other 46 isolates from the screening experiment are prototrophic reversions. The reactions of one of them were investigated. This isolate did not differ from the wild type concerning the response to the B_6 vitamins.

In order to ascertain unequivocally the heritability of the acquired pyridoxal-less character, the strain W 366-10-1 was crossed to a wild type strain. Any possible interference of changes that might have occurred in the stock culture of the mutant strain was excluded by using a monoconidial mycelium as inoculating material in the cross. The result of the intercross is presented in Table 3. Totally 66 germinating ascospores were isolated. Identification of the mycelia emanating from these revealed 24 mutants. These mutants were then tested on minimal medium with 0.3 μ mol pyridoxal per 10 ml. Out of the 24 mutants 10 proved to be pyridoxal-less by responding to this amount of pyridoxal. The remaining 14 were pyridoxamine-less. Two of the mutants from each group were analyzed in shaken tubes for their quantitative reaction towards pyridoxal. The test revealed that the strains were identical with the earlier recognized types, pyridoxal-less and pyridoxamine-less.

Thus the pyridoxal-less character is evidently heritable. The fact that pyridoxamine-less strains are crossed out in the experiment shows that the gene responsible for this character, called *pdxam*, has not undergone mutation. Apparently the ability to grow on pyridoxal medium results from a mutation in a gene separate from *pdxam*. The approximately 1 : 1 ratio between pyridoxal-requiring and pyridoxamine-requiring strains in the mutant offspring shows that the acquired pyridoxal-less character is the result of the action of two genes, *pdxam* and a gene that does not seem to be linked either to *pdxam* or to the mating type locus. The action of this second gene is to restore the capability of the fungus cell to utilize pyridoxal. Mutant genes with the power of relieving metabolic blocks wholly or in part are termed suppressors (Wagner and Mitchell 1955). This gene was therefore

Table 4. Result of a cross between a prototrophic reversion of pyridoxal-less, W 366-10-1-R1 (+) and W 233-2-3 (—), pyridoxamine-less, on the one hand and W 366-10-14 (—) pyridoxamine-less on the other.

Strains	Number of ascospores isolated in f_1			
	Total	Wild type	Pyridoxal-less	Pyridoxamine-less
W 366-10-1-R1 \times W 366-10-14	71	46	14	11
W 366-10-1-R1 \times W 233-2-3	32	20	7	5

called *su-pdxam*⁺ and the allele responsible for the pyridoxal-less character *su-pdxam*.

Synthesis of pyridoxal-less strains. According to the argument presented above the allele *su-pdxam* should be found in about half of the wild type strains obtained from the cross of pyridoxal-less to wild type. The next experimental step would be to combine one of the *su-pdxam*-carrying wild type strains with a pyridoxamine-less strain. The mutant progeny could be expected to contain pyridoxal-deficient types as well as pyridoxamine-deficient ones. In order to investigate the possible phenotypic effect of the suppressor-gene 21 of the wild type strains from the intercross W 366-10-1 to wild type were tested in a preliminary experiment in shaken tubes supplied with 0.3 mmol pyridoxal or the same amount of pyridoxine. Conidia for inoculation were taken directly from the stock culture agar tubes. Growth started more or less simultaneously in all cases. Thus, qualitatively at least, no observable differences could be noted. The suppressor gene does not seem to have any apparent phenotypic effect in combination with *pdxam*⁺ and could not be detected in the wild type. It was therefore decided to use a prototrophic reversion of W 366-10-1 in a cross to pyridoxamine-less. Such a reversion could be supposed to carry the allele *su-pdxam* of the suppressor gene together with the wild type allele, *pdxam*⁺.

For this purpose a spontaneous reversion was selected for a cross, *viz.* W 366-10-1-R1 (+). Genetic analysis of reversions to prototrophic growth habit of biochemical mutations in *Neurospora* (Giles 1951) and *Ophiostoma* (Zetterberg, personal communication) have shown that two mechanisms may be responsible for the phenomenon, *viz.* true reverse-mutations and reversions due to suppressor-mutations. In order to rule out the possibility that a suppressor was responsible for the reversion to wild type, the reverted strain was crossed to wild type. From this intercross 33 isolates were obtained and identified. All these were prototrophic when tested in minimal medium plus pyridoxine at 30°C. It can thus be inferred that the prototrophy was conditioned by a reversion of the mutated gene (*pdxam*) to its wild type allele (*pdxam*⁺). (There is a remote possibility that a suppressor very closely linked

Table 5. *The effect of pyridoxine on the growth of W 366-10-1, pyridoxal-less, at 20°C.*

mmol pyridoxine per flask	Dry weights of mycelium after 6 days
1	6.9
10	12.5
100	13.1

to *pdxam* was operating. Such a mechanism would probably not interfere very much in the experiments below, however, since it would in most cases follow *pdxam* in meiosis.)

The reverse-mutation was also analyzed for its quantitative growth response to pyridoxine, pyridoxal, and pyridoxamine in a shaken tube experiment at 30°C. The extinction values showed that the isolate was identical with the wild type in this respect. Thus the suppressor had no apparent effect on the utilization of the vitamins in the presence of *pdxam*⁺.

An intercross was then made between this reversion and W 366-10-14 (—), pyridoxamine-less, a substrain of W 366-10. The result of this experiment is presented in Table 4. Out of 71 isolated 1-ascospore mycelia 46 were prototrophic. Theoretically the segregation between the wild type and the mutants should be 1:1. It has frequently been observed, however, that in mutant-wild type crosses the number of wild type mycelia exceeds that of the mutants. This is probably due to the fact that wild type spores germinate faster than the mutants. The rest of the isolates were mutants. They could be classified in the two groups pyridoxal-deficient (14) and pyridoxamine-deficient (11). The result of the cross is completely in accordance with the suppositions arrived at. The reversion W366-10-1-R1 is a carrier of *su-pdxam*, and this gene segregates freely in relation to the reverted form of *pdxam*, thus forming the expected double mutant *pdxam*, *su-pdxam*. Half of the wild type should be identical with normal wild type (*pdxam*⁺, *su-pdxam*⁺) and half with the reversion (*pdxam*⁺, *su-pdxam*). The correctness of this prediction seems beyond doubt, although the wild types obtained in the cross have not been analyzed in this respect. An intercross between the R1 strain and the other pyridoxamine-less mutant, W 233-2, was also made. It resulted in the synthesis of 7 pyridoxal-less out of 12 mutants. (See Table 4.) One of the pyridoxal-deficient isolates from this experiment was studied regarding its reaction towards pyridoxal at 30°C. The growth values showed that the type behaved identically with W 366-10-1 under these conditions. Pyridoxine was inactive and inhibited growth. The inhibitory ratio for total inhibition has not yet been determined but is probably the same as for W 366-10-1.

The effect of temperature on the growth of pyridoxal-less strains. As was shown above, one of the pyridoxamine-requiring strains, W 366-10, was

able to grow in pyridoxine-supplemented medium at 20°C, while the other strain, W 233-2, did not have this property. It was naturally of interest to compare the pyridoxal-deficient strains derived from these two pyridoxamine-deficient ones in this respect as well. Both pyridoxal-less types were therefore tested in flasks at 20°C in minimal medium supplied with pyridoxine 1.0, 10.0, and 100 μ mol per 20 ml. medium. In Table 5 the results for W 366-10-1 are compiled in the form of dry weights after incubation for 6 days. It is evident from the positive effect of the vitamin that the double mutant *pdxam*, *su-pdxam* like its parent strain, *pdxam*, *su-pdxam*⁺, is dependent on temperature. In addition, if the quantitative data for the amount of mycelium produced are compared with those obtained from the unsuppressed mutation (Table 2) it is found that both strains are almost identical in their response to pyridoxine. Thus, at 20°C the metabolic block is partly relieved also for the pyridoxal-deficient strain. On the other hand the fact that both the W 366-10, pyridoxamine-less, and W 366-10-1, pyridoxal-less, strains react similarly towards pyridoxine leads to the conclusion that the suppressor does not have any phenotypic effect on the utilization of pyridoxine under these conditions either.

The pyridoxal-requiring isolates obtained from the intercross R1 \times W 233-2 were also tested at 20°C as above. None of five pyridoxal-deficient substrains derived from this cross was stimulated by pyridoxine at this temperature. These isolates thus reacted identically with the original mutation W 233-2. It is concluded that in this case, too, the suppressor gene lacks phenotypic expression as regards pyridoxine metabolism.

Conclusions

The two pyridoxamine-deficient mutants, W 233-2 and W 366-10, discussed in this paper are derived from the pyridoxine-heterotrophic wild type of *Ophiostoma*. It has been shown that an acquired character of pyridoxal-heterotrophy, *i.e.*, the capacity of also utilizing pyridoxal, in an isolate, W 366-10-1, from one of the pyridoxamine-heterotrophic mutants, W 366-10, was heritable. A search for pyridoxal-deficient types from a conidial population of W 366-10 revealed that pyridoxal-less conidia existed in a low frequency in such a population. They are apparently formed by spontaneous mutation, and may be enriched by selection in pyridoxal-containing, pyridoxamine-free medium.

The outcrossing of pyridoxamine-deficient strains when a pyridoxal-deficient strain was mated with wild type indicated that a suppressor gene is responsible for the character. The existence of this suppressor gene was

proved conclusively when pyridoxal-deficient strains were synthesized by combining W 233-2 and W 366-10 with a prototrophic reversion of W 366-10-1, pyridoxal-less.

The suppressor has no observable phenotypic effect except that of changing the pyridoxamine-heterotrophy into a pyridoxal-heterotrophy.

The pyridoxamine-heterotrophic strains have also been compared with regard to their physiological phenotype. It was found that one of the mutants, W 366-10, is temperature-sensitive. In contrast to the other mutant, W 233-2, it also responds to pyridoxal and pyridoxine at 20°C. At this temperature the mutant block is relieved, but not completely, since wild type growth habit is not acquired.

The result of an intercross between the mutants indicates that the mutated genes are non-allelic and closely linked, or possibly pseudo-alleles of the same gene.

Spontaneous mutations to pyridoxal-heterotrophy have only been found in one of the mutants, W 366-10. This fact might be taken as an indication of a difference in mutability in the two strains of the suppressor gene which has been found in the pyridoxal-less isolate. Similar observations have previously been made in *Ophiostoma*. Zetterberg (personal communication) found that the induced increase in the frequency of backmutation of a gene conditioning uracil-heterotrophy of a uracil-methionine-less mutant was larger in the double mutant than in the uracil-less one (carrying the same gene for uracil-deficiency). The phenomenon may be interpreted as an effect of the genetic background, which obviously may be different for the mutants even if the "incrossed" gene giving methionine-heterotrophy is not considered.

Suppressor mutations have previously been observed in several cases in biochemical mutations in *Neurospora*, *e.g.*, methionine-less and inositol-less (Giles 1951) and tryptophan-less (Yanofsky 1952). In these cases the suppressors relieve the requirement of the respective substances. In some cases the mode of growth then agrees with that of the wild type, in others the suppressed strains may still be stimulated by the compound in question. In the latter cases a partial block remains in the biosynthetic path.

The biosynthetic sequence of some steps for vitamin B₆ has been discussed previously (Wikberg 1959), and a tentative scheme was proposed on the basis of the growth requirements of the strains used in the present study. It was suggested that a partial block existed between pyridoxal and pyridoxamine in the pyridoxamine-deficient strains, and that pyridoxine preceded pyridoxal in this sequence. If the action of the suppressor gene found in the pyridoxal-deficient *Ophiostoma* strain is similar to that of *Neurospora* suppressor genes mentioned above, the pyridoxal-deficient strain might be looked upon as a

partial reversion of the original block in W 366-10. However, the fact that the pyridoxal-requiring strain seems to have a complete block between pyridoxine and pyridoxal argues against this assumption.

It therefore seems that other explanations must be looked for. In *Neurospora* it has been found that certain suppressor genes are able to modify the expression of an originally mutated gene in such a way that the metabolic block seems to have moved from one position to another in the same metabolic sequence. Thus one suppressor has been found which in an ornithine-deficient strain has the ability to alter the growth requirements of this strain in such a way that the double mutant appears to be blocked *after* ornithine (Mitchell and Mitchell 1952). Tryptophan-deficient strains in *Neurospora* provide similar evidence for the action of such modifying genes (Haskins & Mitchell 1952; Newmeyer & Tatum 1953). Applying the same reasoning in the present case, the *Ophiostoma* suppressor may be imagined to have moved the original block one step backwards in the metabolic path, thus establishing a block between pyridoxine and pyridoxal instead of between pyridoxal and pyridoxamine.

Still another possibility to explain the action of the suppressor is to suppose an effect of the original mutation on two steps in the same metabolic path via a common reaction, the steps pyridoxine-pyridoxal and pyridoxal-pyridoxamine. The action of the suppressor would then be to relieve only the latter of the two blocks caused by the original mutation. At present no decision can be made between the possibilities outlined.

Summary

Two pyridoxamine-less strains, W 233-2 and W 366-10, of *Ophiostoma multiannulatum* have been compared from physiological and genetical point of view. Both strains give a slow growth response to pyridoxal. For W 366-10 it has been shown that the response to this vitamin is influenced by the genetic background. In contrast to the other mutant, W 366-10 grows with pyridoxine at 20°C but not at 30°C. It is thus temperature-sensitive.

An intercross between the mutants revealed that the genes responsible for the pyridoxamine-less character are non-allelic and closely linked, or possibly pseudo-alleles of the same gene.

A spontaneous mutation to pyridoxal-heterotrophy, W 366-10-1, has been found in a population of W 366-10, pyridoxamine-less. The acquired pyridoxal-less character is conditioned by a suppressor gene. Pyridoxal-less strains have been synthesized by combining W 233-2 and W 366-10 with a prototrophic reversion of W 366-10-1. The suppressor gene has no observable phenotypic effect except the above mentioned.

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On the Kok-Phenomenon in Photosynthesis of Leaves

Interaction of Excess Carbon Dioxide and Temperature on Photosynthesis in Weak Light

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1. Introduction

In more recent literature the curve depicting the dependence of photosynthesis on light intensity [$P=f(I)$] is often referred to in brief as *the light curve*. This curve is usually considered to consist of three parts, i.e., 1) the initial or lower branch showing the rate of photosynthesis to increase rapidly with increasing light intensity, and 2) a pronounced arch bending towards the axis of abscissas and connecting the initial curve part with 3) the upper linear branch, which in most cases, beginning at the point of light saturation, is parallel to the axis of abscissas, thus showing no increase of the photosynthetic rate with increasing light intensity (Rabinowitch 1951, Gabrielsen 1959). In the present paper the interest is first centered on the initial branch of the curve.

In the majority of investigations carried out with chlorophyll-a plants (the photo-autotrophic bacteria appear to constitute a special exception, French 1937, Larsen 1953) it has been found that the initial part of the light curve is in all probability an absolutely straight line. The slope of the curve towards the axis of abscissas is a measure of the rate of the light reactions, and the linearity of the curve may be considered indicative of the fact that within each individual photosynthetic system (leaf, alga thallus, suspensions of unicellular algae) the quantum efficiency, within wide limits, is independent of

light intensity. Not until the illumination reaches a value such as to cause the rate of the light reactions (production of intermediates) in cells situated nearest the light source to exceed that of the dark reactions (consumption of intermediates), does the light curve of the photosynthetic system begin to bend towards the axis of abscissas. The results of careful and exhaustive studies with *Chlorella* suspensions by Emerson and Lewis (1941, 1943) have greatly contributed to the consolidation of the conception of the initial light curve branch as a straight line.

More recently serious objections have been raised against this prevailing conception by Kok (1948, 1949), and by van der Veen (1949). Both of these investigators have published results which differ considerably from the linear plots appearing in the papers of Emerson and Lewis.

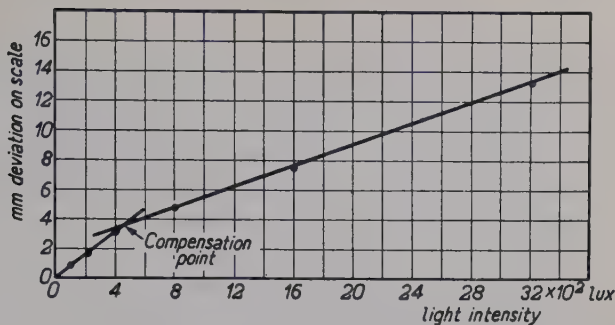
Kok, working with various volumetric measuring techniques, improved from time to time, found that the initial branch of the light curve consisted of two linear components; they formed an apparently sharp break at or close to the light intensity, below which the *Chlorella* suspensions do not accountably take up carbon dioxide from outside, but work photosynthetically with carbon dioxide taken over from respiration (the compensative light intensity, also termed the compensation point). At intensities below that of compensation, or rather below the sharp break, the slope of the curve was in most cases twice that found at higher light intensities above the critical point. In other words, the quantum efficiency in *Chlorella* below the break in the curve was observed to be in most cases twice as high as above it.

Sharp breaks in the initial part of light curves were observed in the main study of Kok, where cells of *Chlorella* were used, as well as in associated studies made with suspensions of cells of *Haematococcus pluvialis* or with *Cabomba* leaves floating on carbonate-buffer. Kok also considered experimental material provided by Kopp (1941, experiments with *Chlorella*) and by Gabrielsen (1947, 1948, experiments with leaves of herbs and trees) to support his conception of the shape of the initial curve.

It is not possible here to quote in detail the explanation offered by Kok of the change in quantum efficiency at the curve break. Briefly summarized, he assumed that the liberation of energy by the green cells during normal respiration ("dark respiration"), at very low light intensities was partly put out of action and replaced by a photochemical process supplying compensating energy to the work of the protoplasm via energy rich phosphorus compounds. The peculiar shape of the initial curve branch might be explained if this particular photochemical process was light saturated at the compensation point of the light curve and if it had a quantum efficiency greater than that of the "true" photosynthesis setting in after the sharp break of the curve.

However, detailed studies by Brackett, Olson and Crickard (1953) indicated

Figure 1. Lower branch of the light curve for tobacco leaves. Experiments in 3.6 vol % CO₂. After van der Veen (1949).



that the manometrically measured respiration values on which Kok based his measurements and calculations of photosynthetic rates were probably not true ones. By using a polarographic method allowing rapid measurements at brief intervals, these three authors were able to show that during intermittent illumination of *Chlorella* (3 minute intervals) the respiration intensity varies periodically due to the influence of photosynthetic products. The waves of the variation occurred with certain retardations relative to the times of onset and cessation of illumination. When calculation of the photosynthetic values were based on mean values for the rate of respiration during the dark periods, it was possible to obtain curves with breaks similar to those found by Kok; this might be considered to be a direct result of the fact that the error committed (by using incorrect respiration values for the calculations) had the relatively greater effect on the results at a low light intensity where photosynthetic values are small. When respiratory values which must be assumed to correspond more closely to the actual intensity of the respiration during the light periods were used, a connection was found between light intensity and photosynthetic rates showing strict linearity for the lower branch of the light curve, in agreement with the results of Emerson and Lewis.

Although it is thus very probable that the breaks occurring in the curves of Kok are due to technical imperfections of the manometric methods used, the break on the initial part of the light curve observed by van der Veen (1949), presents an utterly different case (Figure 1). Van der Veen employed a method (leaves in streaming atmospheric air containing 3.6 vol % CO₂; uptake of CO₂ determined by measuring changes of the heat conductivity of the air stream with Noyons' diaferometer) which should allow for full control of all possible time induced variations of respiratory and photosynthetic values. Measuring errors like those demonstrated in the manometric methods are inconceivable here. In investigations with fragments of tobacco leaves, sharp breaks in the light curves at or near the compensation point were found in all experimental series. On this basis van der Veen accepted the Kok-

phenomenon, as he named it, as being well-founded for use in theoretical considerations concerning the kinetics of the photosynthetic processes.

The present authors have from time to time made attempts to repeat the experiments of van der Veen, using the same method as he. In spite of great efforts we failed to demonstrate the existence of the Kok-phenomenon under ordinary conditions; all light curves made with leaves of various species of plants showed an absolutely linear course within the range of light intensities studied by van der Veen. Hence our results were in complete agreement with the results obtained by Brackett, Olson, and Crickard and by Emerson and Lewis from their *Chlorella* experiments. However, in a final attempt to explain the disagreement between the results of van der Veen and those obtained by us, we varied the external conditions of our experiments, in the first place the temperature. We were encouraged to study the effects of temperature changes by the fact, that no mention was made in van der Veen's paper of the temperature at which he carried out his experiments with tobacco leaves. The fact that the compensation point is found at 400 lux might suggest that the experiments were performed at a temperature lower than 20°C. A number of other experiments (on induction phenomena) discussed in the said article were made at temperatures of from 6° to 0°C. Hence we reduced the temperature to below the 20°, habitually used by us, and, coincidentally, we found in 5.6 vol % carbon dioxide the first indication of what we then considered a break in the lower branch of the light curve.

The following is an account of the results of our studies on the effects of temperature and of carbon dioxide concentration on the initial course of the light curve.

2. Methods and Experiments

The diaferometer method (the gas thermal conductivity method) used in all of our photosynthesis experiments is described in detail by Vejlby (1958). The gas mixtures were prepared in compressed form in steel bombs. In most experiments the measuring accuracy was such that a reading of one mm on the galvanometer scale corresponded to 2.7 ppm of carbon dioxide as calculated per volume (0.00027 vol %).

A water cooled 1500 watt incandescent lamp served as source of light. Changes of the light intensity were produced by varying the distance between the lamp and the leaf container and the intensities were measured in absolute measures by means of a photocell (gcal/dm²h), taking into consideration only radiation below λ 700 m μ (photosynthetically active light). For the type of lamp used 28.6 gcal/dm²h, (λ 400—700 m μ) correspond to about 1000 lux (Gabrielsen 1948).

Most experiments were made with leaves of potato plants (*Solanum tuberosum* L., variety Bintje). The stomata of these plants do not show any reaction following light/dark shifts, and because of this fact their leaves were found to be particularly suitable for diaferometer measurements, where the apparatus in the dark must be

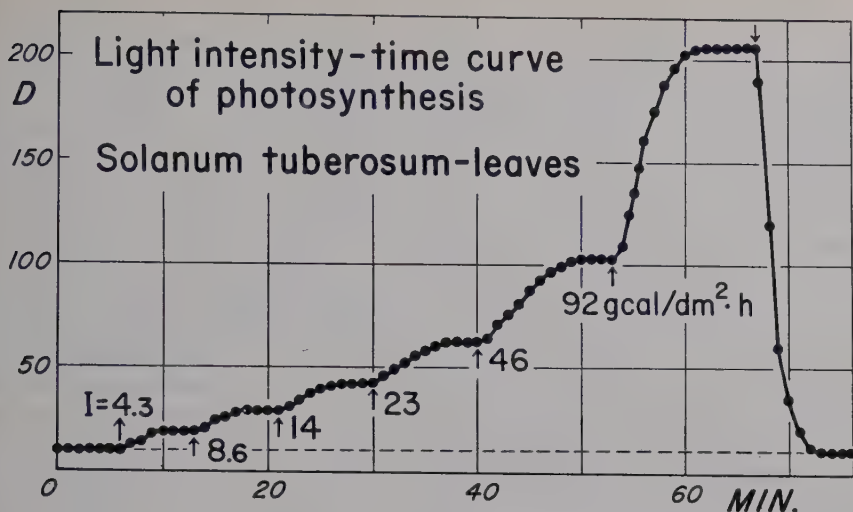


Figure 2. Example of an experimental series. Upward arrows indicate shift in intensity, downward arrows indicate light off. Ordinate photosynthesis in mm galvanometer deviation. Illumination white incandescent light, measurements comprising wave lengths below λ 700 m μ only.

brought into equilibrium at the respiratory level of the leaves before illumination experiments can be carried out. When the water supply was ample, the stomata were found to be fully open (measurements by means of the stomatometer of Boysen Jensen) even after more than 48 hours in the dark. A few experiments were made with leaves of the sugar beet. The stomatal conditions of the sample leaves were controlled before (on cut parts) and after experimental series. Closing of the stomata, which might be feared at high concentrations of carbon dioxide (Linsbauer 1916, Chapman et al. 1924), was not observed at the concentrations used here (up to 20 vol % CO_2). The leaves were supplied with water during their stay in the leaf container by means of small vases.

The arrangement of the experimental series appears from Figure 2. Following the determination in the dark of the slope of the time-respiration-curve (the respiration level) the light was turned on, and when the time-photosynthesis-curve had reached a "steady state" level, another light intensity was introduced, etc. After conclusion of the illumination experiments the slope of the time-respiration-curve was again measured, and if this second slope (which depends upon the "drift" of the apparatus) did not agree with the one measured prior to the experiment series, all of the results was discarded. In Figure 2 the slope of the time-respiration-curve has been corrected to obtain a course parallel to the axis of abscissas. On the basis of the distance between the steady state levels of photosynthesis and that of respiration, relative values might be obtained for the photosynthetic rate at varying light intensities (mm galvanometer reading \sim true photosynthesis). On this basis the light curve of photosynthesis can be drawn.

In contrast to the procedure of van der Veen, we made an attempt to convert the relative values for the photosynthetic activity into absolute ones ($\text{mg CO}_2/\text{dm}^2\text{h}$), by

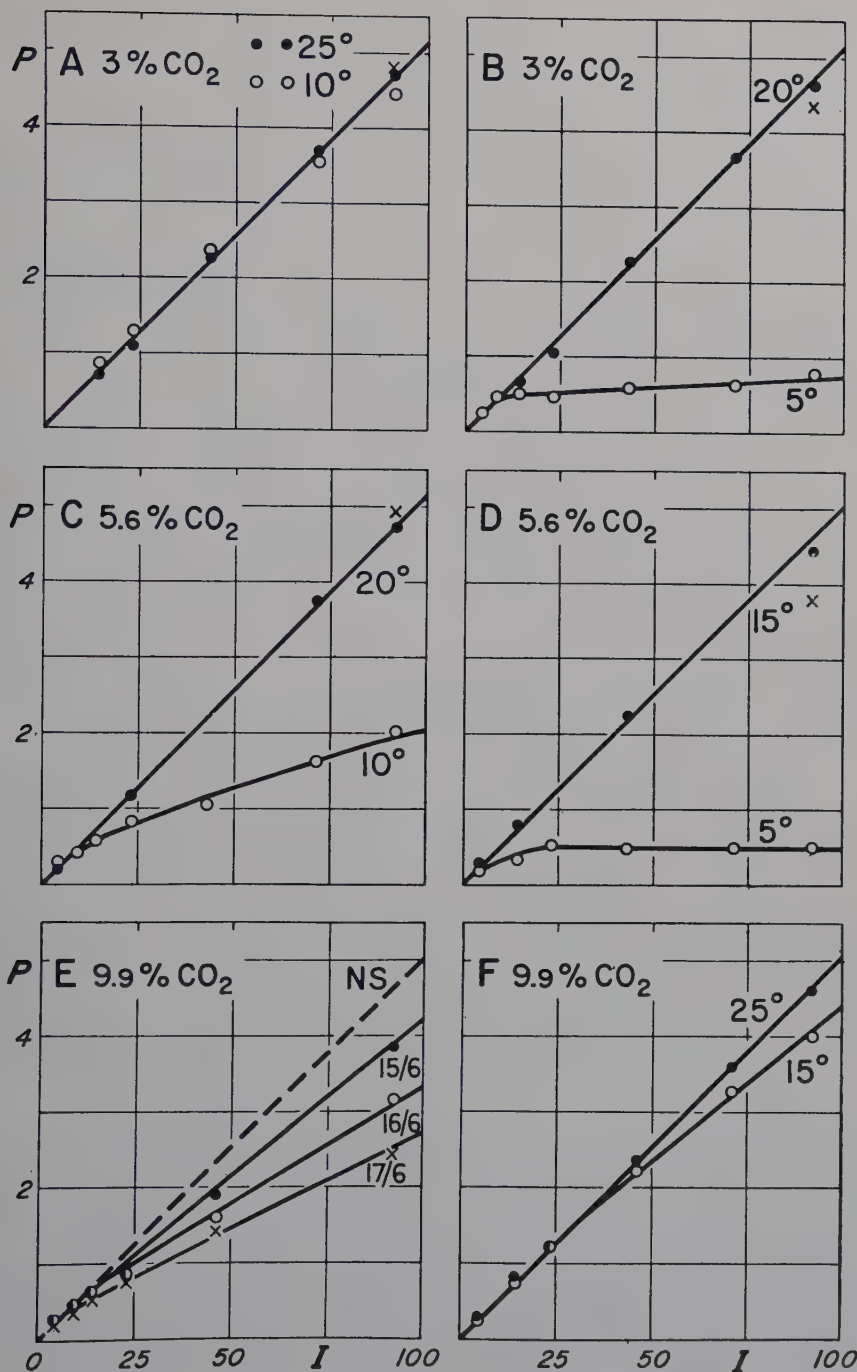
adjusting the galvanometer readings to known changes in the carbon dioxide concentration of the gas stream. It was further necessary to determine the rate of air flow past the plant material. While the former adjustment was relatively easy to make, the latter caused a certain amount of trouble; it was easy to avoid variations in the rate of flow within each individual series of experiments (which is necessary for the proper functioning of the apparatus), but it was not possible to adjust the apparatus to the very same rate of flow day by day, or to measure the changes with a reasonable degree of accuracy. For the presentation of the data (Figures 3 and 4) we therefore chose to correct the linear light curves found under normal conditions to a slope corresponding to a photosynthetic rate of $2.05 \text{ mg CO}_2/\text{dm}^2\text{h}$ at an illumination of $40 \text{ gcal}/\text{dm}^2\text{h}$ (average value for green and dark green leaves containing from 5 to 9 mg of chlorophyll (a+b) per dm^2 , Gabrielsen 1948). All other photosynthetic values outside of linear, normal curves and the values determining the course of these curves were corrected relative to this. The corrections may be considered of minor significance for the discussion later in this paper, except for the fact that it facilitates summarizing the results obtained.

The experiments were made in atmospheric air to which had been added 3.0, 5.6, 9.9, and 20 vol % CO_2 , respectively. A representative sample of the results is presented in Figures 3 and 4.

In air with 3.0 % CO_2 two light curves were measured at 25° and 10°C . Both sets of measurements were made with one individual leaf, separated only by the period of time involved in adjusting the water bath around the leaf container from the high to the low experimental temperature (Figure 3A). The values measured all fall close to a straight line together with the control measurement indicated by a cross. The control measurement was made after the temperature around the leaf container had again been raised to 25° . Both of the curves in Figure 3B were also measured with one experimental leaf. A reduction of the temperature from 20° to 5° causes the light curve to bend relatively sharply towards the axis of abscissas at a comparatively low light intensity, i.e., $10 \text{ gcal}/\text{dm}^2\text{h}$, corresponding to about 300 lux. The control value at 20° , measured after the 5° -series, falls very close to the 20° -curve.

In air with 5.6 % CO_2 , as in the 3.0 %-series, four curves were measured, two and two with the same experimental leaf (Figures 3C and 3D). In contrast to the 3.0 %-series a reduction of photosynthesis occurs in this case already at 10° , where at an illumination at about $12.5 \text{ gcal}/\text{dm}^2\text{h}$, corresponding to about 450 lux, the light curve bends gradually towards the axis of abscissas without actually becoming parallel to it within the range of light

Figure 3. *Light curves for potato leaves.* A, B, C, D, and F separately represent experiments with one individual leaf; constant concentration of carbon dioxide; temperature varied, high at first, low subsequently. Crosses indicate control measurements at higher temperatures, following experiments at lower temperatures. E represents three light curves at 20°C , measured each with its own individual leaf. NS normal slope. P photosynthesis in $\text{mg CO}_2/\text{dm}^2\text{h}$, I light intensity in $\text{gcal}/\text{dm}^2\text{h}$ (λ 400 to 700 μ).



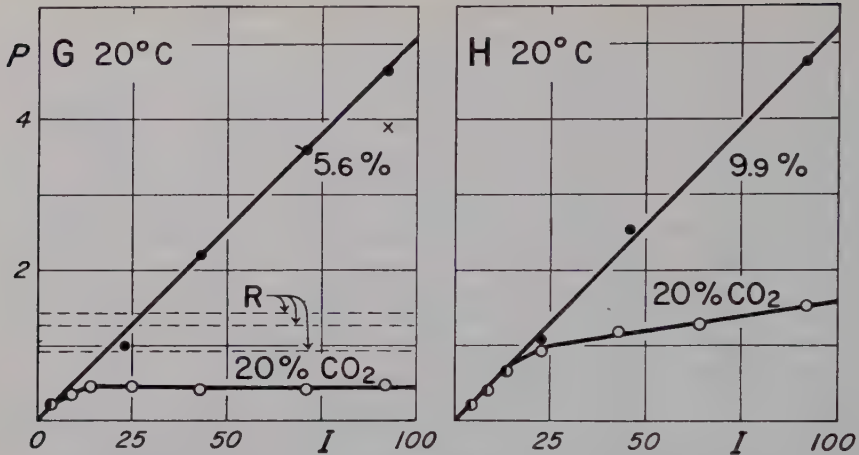


Figure 4. Light curves for potato leaves (G) and beet leaves (H). Experiments at constant temperature and varied concentrations of carbon dioxide, low at first, high subsequently. Cross represents result of control experiment following those at high concentrations of carbon dioxide. *R* shows the mean value, plus the highest and the lowest value for respiratory rates ($\text{mg CO}_2/\text{dm}^2\text{h}$) in a sample of potato leaves similar to those used in the photosynthesis experiments. *P* and *I* as in Figure 3.

intensity investigated. The 5° -curve in Figure D bends around to complete parallelism with the axis of abscissas, in contrast to the 3.0% - 5° -curve.

In air with 9.9% CO_2 an effect may be noticed already at 15° , where the light curve shows a slight break at a light intensity of about $30 \text{ gcal}/\text{dm}^2\text{h} \sim 1050 \text{ lux}$ (Figure 3 F); this is in contrast to both the 3.0 and the 5.6% series. Other curves at 20° for different leaves, measured on different days show similar breaks (Figure 3 E), although at lower light intensities than in the 15° -curve in Figure 3 F. The slopes of these 20° -curves, however, were not determined with the same accuracy as was that of the 15° -curve, as only one light curve was measured for each leaf, and hence no comparison and correction to the slope of a normal light curve for the experimental leaf itself was possible.

In air with 20% CO_2 a considerable reduction of the photosynthesis of potato leaves was found (Figure 4 G). In this case, the experiments were carried out in a way different from that applied before, measurements being made in two different concentrations of CO_2 with the same sample leaf at the same temperature (20°). First one light curve was measured in an air stream containing 5.6% CO_2 , changing subsequently to a stream with 20% CO_2 for the measurement of another light curve; a final control measurement was made in the former concentration. The 5.6% - 20° -light curve constitutes a

straight line, as in Figure 3 C, whereas the 20 % CO_2 -light curve bends and reaches a saturation point at 15 gcal/dm²h.

In Figure 4 G lines are added, showing the mean value as well as the highest and the lowest value for the respiration of potato leaves (mg CO_2 /dm²h). The values were obtained from 10 measurements with different leaves made in air above a solution of barium hydroxide (3 hour experiments, for the method used see Boysen Jensen 1932 or Gabrielsen 1940). The reducing effect on photosynthesis of the high concentration (20 %) of carbon dioxide is so pronounced that even the reassimilation of respiratory carbon dioxide is considerably inhibited.

Some experiments with beet leaves made in air containing 9.9 % and 20 % CO_2 (Figure 4 H) show that in this case the inhibiting effect on photosynthesis of the high concentration of carbon dioxide is not quite as pronounced as in the case of potato leaves. Within the range of light intensities studied the 20 % CO_2 -light curve does not reach complete light saturation.

3. Discussion and Conclusions

The experiments clearly show that high concentrations of carbon dioxide have distinct inhibitory effects on the process of photosynthesis, affecting the course of the light curve, even at low light intensities. The interaction with temperature is summarized in the following table:

CO_2 (vol. %)	Effect at °C. (light curve more or less sharply bent, Fig. no. in brackets)	No effect at °C. (light curve linear, Fig. no. in brackets)
3.0	5° (3B)	10°, —, 20°, 25° (3A, B)
5.6	5°, 10° (3D, C)	15°, 20° (3D, C, 4G)
9.9	15°, 20° (3E, F)	20°, 25° (4H, 3F)
20.0	20° (4G, H)	?

It is evident that the inhibition 1) at uniform temperatures increases with increasing concentrations of carbon dioxide and 2) at uniform concentrations of carbon dioxide is brought to a cessation with increasing temperatures. Further, a more detailed analysis of the curve material shows that the cessation of inhibition occurs the later within the range of temperature the higher the concentration of carbon dioxide surrounding the leaves. The facts stressed here suggest that adsorptive forces come into play. Adsorptive phenomena are known to produce increasing effect with increasing concentrations at the same temperature, and decreasing effect with increasing temperatures at the same concentration (cp., e.g., Daniels 1948). It thus appears to be fairly certain that the inhibitory effect on photosynthesis produced by excess carbon dioxide consists in a blockage of active surfaces or of phase boundaries, and that hence it is of narcotic nature. The latter conclusion is emphasized by

the fact that the inhibition in our investigations, as in all truly narcotic phenomena, is fully reversible (observe the control measurements in Figures 3 B, C, D, and particularly in Figure 4 G).

The narcotic nature of the carbon dioxide poisoning in its effect on photosynthesis has been suggested by several authors (Blackman and Smith 1911, Stiles 1925, Spoehr 1926, Singh and Kumar 1935, Rabinowitch 1945), but as far as we know, it has not previously been proved by direct experiments as in the present study.

Figures 3 F and 4 H in particular give accurate information with regard to the mechanism of the inhibition. Here the curves for the narcotized leaves are distinctly seen to follow the curves for the unaffected leaves in their lower course indicating the photochemical partial processes of photosynthesis (the light reactions) to be completely unaffected by the narcotic effect of the carbon dioxide molecules. It seems to be absolutely certain that only the dark reactions, or factors regulating work or working conditions directly related to these, are blocked to a greater or lesser extent.

A more extensive narcotic effect occurs at low temperatures (see the 5.6 %/5°-curve in Figure 3 D) or at high carbon dioxide concentrations (observe the 20 %/20°-curve in Figure 4 G). In these cases nearly all of the dark reaction mechanism has been inactivated, and the curve soon (at a very low light intensity) reaches a point of light saturation where, because of blockage, the dark reactions are able to convert only relatively small amounts of the intermediates produced by the light reactions.

A complete blockage, i.e., a complete suspension of the photosynthetic processes, does, however, not occur at the temperatures and carbon dioxide concentrations applied in the present investigations.

A slighter narcotic effect of excess supply of carbon dioxide occurs in potato leaves at temperatures higher than 5° and at carbon dioxide concentrations below 20 per cent. In the 5.6 %/10°-curve (Figure 3 C) the narcotization does not lead to complete light saturation, and the curve does not become parallel to the axis of abscissas within the range of light intensities studied; possibly the increasing light intensities have a heat effect counteracting the adsorption effect. The least narcotic effect is observed in the experiments with 9.9 % CO₂ in 15° and the 20°-curves (Figures 3 E, F). Here it is justified to a certain extent to draw the curves as consisting of two linear parts forming a sharp break (van der Veen-curves). Presumably, though, the true curves in such cases are very slightly bent arches, which subsequently, outside of the range of light intensities studied, are leading to light saturation at photosynthetic rates lower than those in the case of unaffected leaves.

All of the facts suggest that excess supplies of carbon dioxide produce a

narcotic effect on photosynthesis, an effect setting in gradually with increasing concentrations of carbon dioxide and decreasing temperatures. The gradual increase of the effect manifests itself by an increasingly extensive inactivation of the dark reaction mechanism, causing the saturation point of the light curve (the light saturation point) to be shifted towards still lower photosynthetic rates and still lower light intensities with increasing concentrations of carbon dioxide or decreasing temperatures.

A consequence of the above explanation of the inhibition phenomena is the fact that the narcotic effect (the ratio of the photosynthetic rate in narcotized leaves to that of unaffected leaves) for identical concentrations of carbon dioxide and identical temperatures, at high light intensities within certain limits, need not necessarily vary with the light intensity; i.e., in the cases where the upper parts of the light curves for unaffected and for narcotized leaves are parallel. At light intensities just below this range of parallelism (below the saturation point for unaffected leaves) the narcotic effect will decrease more and more with decreasing light intensities to become zero at the point where the light curves for the narcotized and the non-narcotized leaves diverge.

A further proof of the probability of the conclusions drawn is found in studies by Ballard (1941) and by Livingston and Franck (1940). In leaves of *Ligustrum*, Ballard was able to demonstrate an inhibition of the photosynthetic activity when the surrounding concentration of carbon dioxide was increased to from 2.0 to 2.5 % (Blackman's palladium black method in a hydrogen atmosphere). The inhibition, however, was found exclusively at low temperatures (6°C). At 16° no reduction of the photosynthetic rate could be observed, even in 5 % CO₂. At 6° the reduction was greater at 36,000 lux than at 2000 lux; the latter intensity presumably lies below the point of light saturation for unaffected leaves. Livingston and Franck observed a similar interaction between the inhibition of photosynthesis and the light intensity to exist in Warburg experiments with leaves of *Hydrangea otaksa* in 20 % CO₂ at 20° (cut segments of leaves in the gas phase of the apparatus, relative measurements of photosynthetic rates and light intensity). All of these observations are supporting our data.

The investigations by Ballard suggest that cotyledons of *Ricinus* plants show greater sensitivity to increases of the concentration of carbon dioxide than do leaves of *Ligustrum*. In some but not in all cases a slight decrease of the photosynthetic rates at 16°C occurred in the *Ricinus* leaves when the concentration was increased to between 4 and 5 %, whereas the *Ligustrum* leaves in no case reacted by reduced photosynthesis upon this combination of temperature and concentration. In our experiments as well differences in sensitivity were observed. Beet leaves are slightly more hardy than are leaves

of potato plants (Figure 4 G, H). Furthermore, the combination of 9.9 per cent of carbon dioxide and a temperature of 15 to 20°C appears to be a critical one at which even leaves of the same species show different sensitivities (Figure 3 E, F).

On the whole, the sensitivity towards high concentrations of carbon dioxide appears to vary to a great extent from species to species, provided all data given in the literature are equally creditable. In *Spironema fragrans* Jaccard and Jaag (1932) found photosynthesis to proceed regularly in an atmosphere with initial concentrations of 20 per cent of carbon dioxide; the experiments were carried out at high temperatures (30 to 35°C), which may to some extent account for the absence of a narcotic effect. A concentration of 30 per cent of carbon dioxide had a harmful effect on the leaves. In leaves of *Hydrangea* Livingston and Franck (1940) found a very considerable reduction of the rate of photosynthesis at a concentration of 20 % CO₂ (20°). Godlewski 1874 and Kreusler 1885 found in photosynthesis experiments, comprising several species of plants, an inhibition at concentrations of between 7 and 10 per cent (high light intensities; temperatures mostly above 25°). A particularly high degree of sensitivity was observed by Singh and Kumar (1935) in experiments with leaves of radish plants, in which photosynthesis was suspended completely at a concentration of about 6 per cent of carbon dioxide (28,650 lux, 29°). Strangely enough the sensitivity was lower at higher light intensities (68,760 lux), where at 29° a very high rate of photosynthesis was found in 6 % CO₂. The observation made by Singh and Lal (1935) that photosynthesis in leaves of the sugar-cane at low light intensities (30°), is reduced already upon the increase of the CO₂ concentration to 0.12 per cent, is probably due to imperfections in the method used. A particularly low degree of sensitivity (high tolerance) has been demonstrated to exist in submerse aquatic plants such as *Helodea* and *Fontinalis*. In experiments at relatively low light intensities and at 23 to 28°, Blackman and Smith (1911) failed to observe any inhibition of photosynthesis in these plants, even when the concentration of carbon dioxide in the water was increased to 0.54 g/100 ml, corresponding approximately to an atmosphere containing 30 per cent of carbon dioxide.

It has been emphasized above that the intensity of the narcotic effect of carbon dioxide depends to a very high degree on external factors such as temperature and light intensity. Even bearing this in mind, the variations quoted of the sensitivity to excess supplies of carbon dioxide seem to be very improbable. More research within this field would be desirable. A problem which should be remembered in this connection was raised by Livingston and Franck in the paper, which has here been repeatedly quoted (1940). They were able to demonstrate that a stepwise increase of the concentration of

carbon dioxide around continuously illuminated leaves of *Hydrangea* caused an increase in their ability to photosynthesize in high concentrations of carbon dioxide. Hence it should be possible to adapt leaves to greater resistance towards narcotic effect. This phenomenon must necessarily be considered, when comparing different species of plants with regard to their abilities to photosynthesize in excess carbon dioxide. It would be of interest to repeat the adaptation experiments of Livingston and Franck with a method even more suited for the study of photosynthesis in leaves than is the Warburg-technique.

With regard to suggestions previously made concerning the mechanism of the effect of excess carbon dioxide on the processes of photosynthesis, it may be mentioned that Livingston and Franck (1940) as well as Ballard (1941) report this factor to have an inhibiting effect which may qualitatively be compared to the effect of hydrogen cyanide.

There is some truth in this, both factors affecting the light curve in the same way, *i.e.*, lowering its upper branch and shifting its saturation point towards lower light intensities. But with this similarity in the course of the light curve, the similarity between the inhibition by excess CO_2 and the inhibition by HCN seems to be exhausted. The HCN effect is directed towards enzymes containing metals; these enzymes are rendered inactive and irreversibly removed from work, and as the dark reactions (the rate of which determines the ordinate of the upper branch of the light curve) do indeed include several enzyme reactions, the effect of HCN may be explained as being an inactivation of photosynthetically working enzyme molecules in stoichiometric proportions. The case of the excess- CO_2 factor is different, since the narcotic effect demonstrated can hardly be expected to affect enzyme systems in a way comparable to that indicated above. In this case, it would seem reasonable to assume that the many different enzymes involved in the dark reactions is distributed in specifically coordinated working groups, action-rings, each individual group being able to utilize the carrier bound products of the light reactions for the binding and reduction of CO_2 -molecules and for the synthesis of the final products of photosynthesis. Each of these action-rings may be imagined to be placed closely up against pigment molecules (chlorophyll-a) on the phase boundaries in the grana of the plastids, in such a way that small centres for the cooperation between the light and the dark reactions are found scattered on the surfaces. If one by one these centres were blocked by adsorbed excess- CO_2 , the narcotic effect of this factor could be explained in agreement with the course of the light curve. According to this theory, then, the excess- CO_2 factor would be similar to the HCN factor in its effect, only not by inactivating individual enzyme mole-

cules, but by gradually removing groups of enzymes from the photosynthetic mechanism.

Finally we should like to offer a few comments on the problem initiating the present study. Our results show the initial branch of the light curve for chlorophyll-a plants still must be considered linear, as has previously been assumed, and that the curves forming a break close to the compensation point, as those observed by van der Veen, are hardly normal phenomena. Our results cannot support the conception of the existence of a Kok-effect in photosynthesis of leaves, either, not even under special abnormal conditions of photosynthesis. It would be reasonable to assume, although there is no direct proof, that the breaks in the curves of van der Veen are due to a beginning CO_2 -narcotization, induced by a low experimental temperature in connection with the relatively high concentration of carbon dioxide used by him (3.6 %). The breaks in the curves are then hardly to be considered real breaks; presumably the curves merely represent ordinary light curves, the bending of which towards the axis of abscissas sets in as a smooth curvature at a very low light intensity, due to the narcotic effect of excess carbon dioxide.

Summary

1. The observations of van der Veen (1949), that light curves for photosynthesis of leaves [$P=f(I)$] exhibit a sharp break (the Kok-phenomenon) at or close to the compensation point, have been checked. The method used was the same as that used by van der Veen.
2. Under normal conditions (20°C , concentrations of carbon dioxide of up to 3.0 vol %) we failed to demonstrate the presence of such breaks in all cases and in all species of plants tested. Throughout the range of light intensities studied by van der Veen, our light curves were found to be absolutely linear, indicating that within wide limits the quantum efficiency is independent of the light intensity. The conception of the existence of a Kok-phenomenon in photosynthesis of leaves (shift of quantum efficiency at the compensation point) cannot be supported.
3. In air with high concentrations of carbon dioxide (3.0 %, 5.6 %) it was possible at 5° and at 10°C to observe light curves bending towards the axis of abscissas at low light intensities. In air with 9.9 % and 20 % CO_2 the curves were observed to bend at 20° . Some of the bent curves show the presence of breaks, similar to those found in the curves of van der Veen.
4. The photosynthesis reducing effect of excess carbon dioxide thus demonstrated increases with increasing CO_2 -concentrations and decreases with

- increasing temperatures. It is fully reversible and is suspended at higher temperatures or at lower concentrations of carbon dioxide. It may be compared to a narcotic poisoning.
5. The narcotic effect affects the dark reactions of photosynthesis exclusively; the mechanism of the narcotization is discussed.
 6. The occurrence of breaks in the light curves of van der Veen may possibly be explained by the fact that a low temperature was used in the experiments in connection with a relatively high concentration of carbon dioxide (3.6 ‰).

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Effect of Time Factor on the Stimulation of Pollen Germination and Pollen Tube Growth by Certain Auxins, Vitamins, and Trace Elements

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Introduction

In a recent study on the physiology of the pollen grains of arecanut (*Arèca catechu* Linn.), Raghavan and Baruah (1956 b) have emphasised that certain auxins like 3-indoleacetic acid (IAA), 3-indolebutyric acid (IBA), 3-indolepropionic acid (IPA) and 2-naphthaleneacetic acid (NAA), vitamins like para-aminobenzoic acid (PABA), ascorbic acid (AA) and inositol and trace elements like boric acid (H_3BO_3), cobalt chloride ($CoCl_2$), lithium chloride ($LiCl$), manganese sulphate ($MnSO_4$), ammonium molybdate [$(NH_4)_2 MoO_4$], zinc sulphate ($ZnSO_4$) and auric chloride ($AuCl_3$) exert a growth promoting action, the stimulation being manifest in an increase in the extent of germination of the pollen grains and enhanced rate of growth of the pollen tubes. The positive action of the stimulants on the pollen grains has been explained in relation to the auxin/vitamin/trace element balance maintained by these substances naturally occurring in the pollen grains, supplemented by that in the external medium and upon this assumption the theory of pollen tube growth by stimulants has been founded (Smith 1942, Raghavan and Baruah 1956 b). Considering the current discussion on the physiological activity of the growth promoting substances, it is of interest to ascertain whether the stimulation by the test substances is specifically of the nature of accelerating

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the germination of the pollen grains or the growth of the pollen tubes. So far all the experimental data on the activity of the growth promoting substances have come mostly from studies on the elongation of coleoptiles and root tips. Several investigators (summarised in Visser 1955) have pointed out the responses of pollen grains to certain stimulants and even in those cases where the positive sensitivity of a particular substance has been established beyond doubt, there is little information concerning the nature of activity of the substances responsible for the stimulation. The present paper has combined previous observations by us on pollen germination and pollen tube growth induced by certain chemical substances with those under the same stimulation determined in a wide range of time after sowing. A brief discussion of the specific role of the substances in stimulating pollen germination and subsequent growth of the pollen tubes is also given.

Material and Methods

Pollen grains used in these studies were collected from arecanut palms under cultivation at Gauhati, India. In choosing the pollen grains for germination studies, fresh flowers in the late bud stage were brought to the laboratory in the evenings so that the flowers were at the proper stage for use the next day. Because of the variability of the samples from different flowers or from the same flower, pollen grains used in any one experiment were collected from different anthers, mixed and used as an inoculum.

The different series of experiments involving all the test substances, and forming one replication were performed with flowers from a single spadix. The pollen grains were germinated in a Van Tiegham Cell by the hanging drop technique at $28 \pm 1^\circ\text{C}$. and about 90 % humidity. At suitable intervals during the growth period, the slides were removed and the number of pollen grains germinated and the extension in pollen tube growth measured. More than 100 pollen grains were counted to determine the extent of germination. The data for the percentage of germination are the average of replicates, whereas for the measurement of the extension in growth of the pollen tubes, a number of pollen tubes were marked on the cover glass with ink dots and kept under observation. Such a procedure was adopted since in a culture all the pollen grains did not germinate to produce tubes simultaneously. Their average growth rate in two replications was later computed. Extension in growth rate was measured with an ocular-micrometer at different periods varying from 0—24 hours from the time of sowing the pollen grains. An average elongation of the pollen tubes which is greater by $10\ \mu$ than that occurring in the control was arbitrarily considered to be significant. Sterility in the cultures after 12—24 hours was not possible, but the growth of bacteria and fungi was kept down to negligible proportions by using sterilised water, glasswares and instruments.

Germination studies were carried out with 14 test substances, comprising 4 auxins (IAA, IBA, NAA and IPA), 3 vitamins (PABA, AA and inositol) and 7 trace elements (H_3BO_3 , CoCl_2 , LiCl , MnSO_4 , $[(\text{NH}_4)_2\text{MoO}_4]$, ZnSO_4 and AuCl_3). Counts were made on the pollen grains germinating in a wide range of concentrations of the substances

which was added to a basal medium of carbohydrate (sucrose, 0.75 %). Growth responses determined in sucrose without the test substances served as control. The number of slides with germinating pollen grains that can conveniently be handled in one shift is limited and so the determinations with each substance were broken up into a series of individual experiments in which the effect of a particular substance in different concentrations was studied separately. Controls were run with each set.

Results

The pollen tubes grew for periods extending up to 24 hours with a constant or increasing growth rate under the influence of the particular medium. Three phases can be broadly distinguished in the germination of pollen grains and growth of pollen tubes either in the basal medium alone or with the addition of test substances. There is an initial lag phase lasting 1—2 hours from sowing, followed by a period of stimulation extending generally from 3—12 hours. The final phase lasts from 12—24 hours and no appreciable stimulation is recorded during this period. Results of a typical experiment using sucrose only is presented in Figure 1. In the case of germination of the pollen grains, more than 80 % of the total germination was observed during the initial phase and early in the second phase. In the final phase, an increase in the extent of germination by only 2—5 % was noted. The maximum increase in germination (30—51 % of the total) occurred in the second phase, 2—3 hours from sowing. The course of growth of the pollen tubes shows a typical lag phase, followed by a period of uniformly rapid growth. The peak period of elongation was during 2—8 hours from sowing, the time of pronounced stimulation being at 6—8 hours. The period of growth of the pollen tubes persisted up to 24 hours, but no marked increase in length was evident during the final phase. Replications of this experiment gave identical results in respect of the mode of growth of the pollen tubes, the only variations among replications being minor differences in their absolute length.

Addition of test substances caused considerable increase in the activity of the pollen grains, the magnitude of the effect being dependent on the time of treatment and the concentration of the test substances (Figures 2—9).¹ In addition to an overall increase in the germination potential of the pollen grains in lower and optimal concentrations, the stimulation was also characterised by a distinct and rapidly visible increase in the extent of germination during the first hour of sowing. For instance, in non-inhibitory concentrations of CoCl_2 , MnSO_4 , and $(\text{NH}_4)_2 \text{MoO}_4$ the extent of germination in the first hour of sowing was nearly 70—90 % of the total (Figures 7 A, 8 A, 9 A). With IAA, IBA, NAA, H_3BO_3 , LiCl , ZnSO_4 , and AuCl_3 the results were sim-

¹ In order to conserve space, only typical diagrams of each group of substances are given.

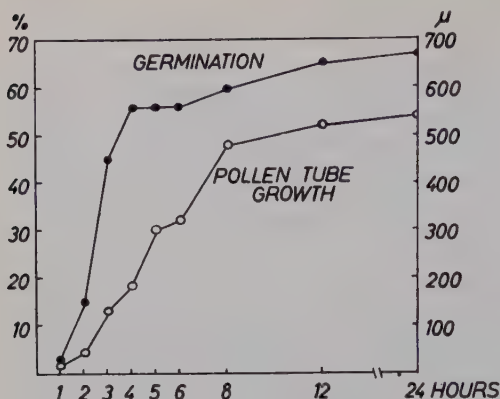
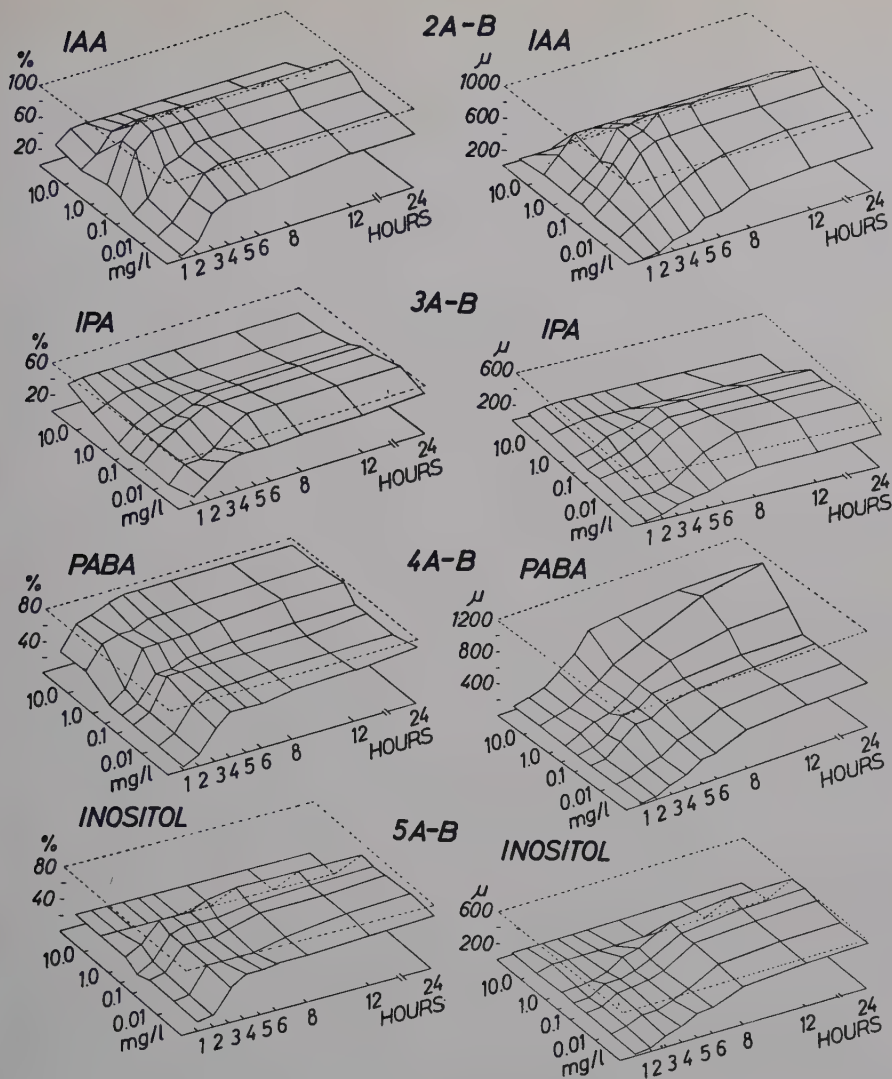


Figure 1. Graph showing the rate of germination of the pollen grains and growth of the pollen tubes in sucrose in relation to time.

ilar, but less spectacular (Figures 2 A, 6 A). In the vitamins, however, no response to non-lethal doses was noted in the initial phase and acceleration in germination was entirely confined to the second phase (Figures 4 A, 5 A). In IPA (0.01—1.0 mg./l.) there was an initial period of inhibition of pollen germination followed by a period of stimulation (Figure 3 A). In lethal concentrations of the substances, inhibition was evident even during the first hour of sowing and was maintained throughout the period under observation. The pollen grains in such cases bloated out to produce vesicular tubes of abnormal shapes, which seldom grew further. With almost all substances, stimulation was confined to the first and early part of the second phase and tended to disappear in the final phase. The horizontal lines in the figures thus show that no further germination or growth was recorded. The behaviour of the pollen grains in PABA and inositol differs in that the maximum germination and pollen tube growth were not observed until 24 hours and thus approaches the condition in sucrose. This was regular in non-lethal doses of inositol and sporadic in PABA; these general features of PABA and inositol effect have been reproduced consistently in replications.

The effect of serial concentrations of IAA and IPA on the growth in length of the pollen tube is indicated in Figures 2 B and 3 B. It is found that the growth of the pollen tubes is entirely confined to the first and second phases. IAA, IBA and NAA stimulate the extension in growth of the pollen tubes at concentrations of 0.01—1.0 mg./l. during the first hour of sowing, the increase in extension being 2—7 % more than in control and was in proportion to the concentration of the auxin. Thereafter, the rate of growth was faster and was significant both in terms of the percentage of the total length and the absolute length. In IPA (0.01—1.0 mg./l.) an inhibition in the growth of the pollen tubes correlated with an inhibition in germination was noted 1—2 hours from sowing, followed by a period of stimulation during 3—8



Figures 2—5. Three dimensional graphs showing the rate of germination of the pollen grains and growth of the pollen tubes in serial concentrations of IAA, IPA, PABA, and inositol in relation to time. Figures 2 A, 3 A, 4 A, 5 A: Germination. Figures 2 B, 3 B, 4 B, 5 B: Pollen tube growth.

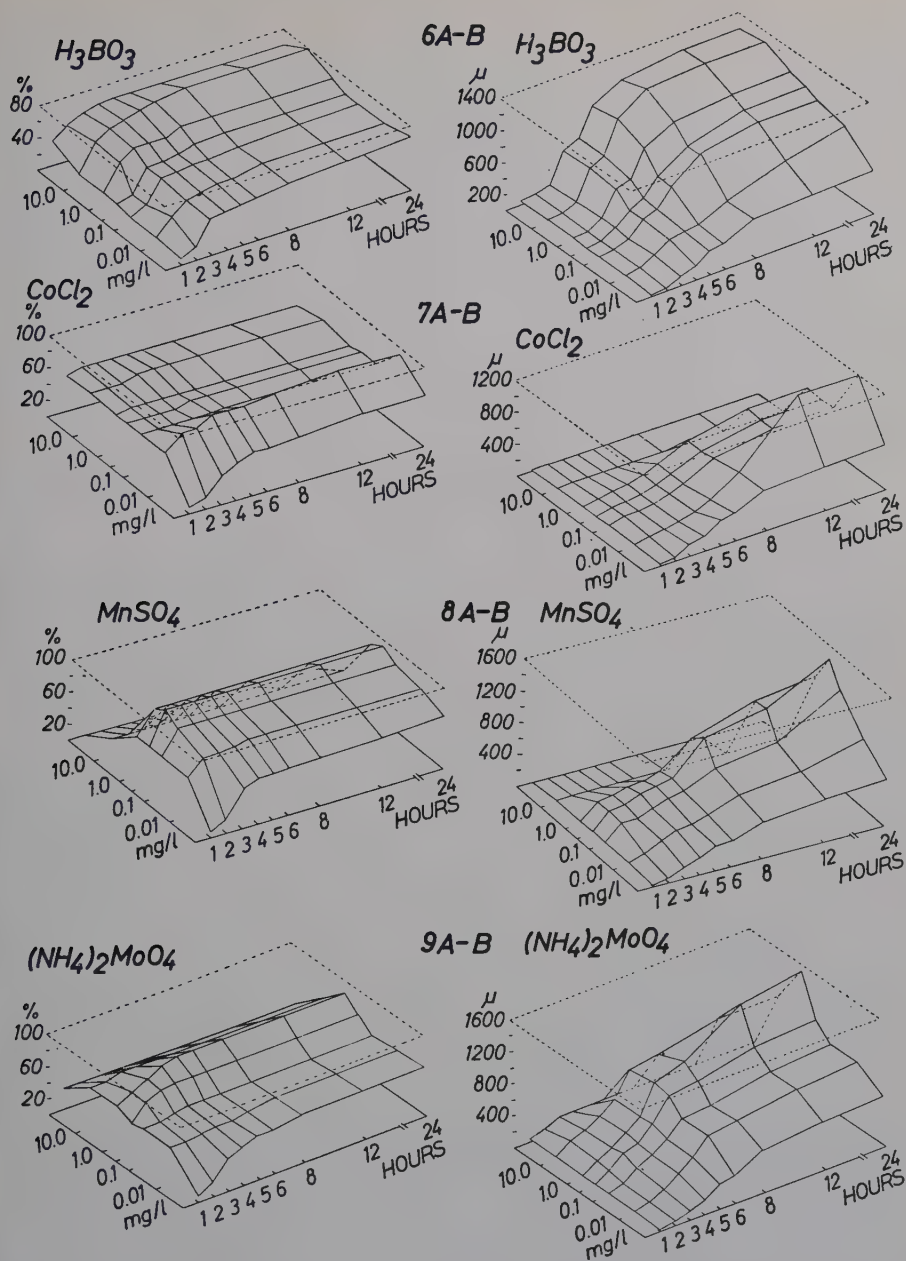
hours. The period of stimulation in IPA was sharp and comparatively short-lived; except in these respects, the results with IPA were similar to those obtained with other auxins.

With optimal concentrations of IAA, IBA, IPA and NAA the promotive

influence was particularly evident during the second phase with peaks at 2—4, 6—8, 6—8 and 3—4 hours respectively. With higher concentrations there was tendency to shift the peak period of stimulation proportionately earlier. For purposes of demonstrating the inhibitory action of higher concentrations of auxins, the cultures need be grown for 2—3 hours, no growth being evident afterwards. However, on a critical examination of the percentage of growth of the pollen tubes in relation to their total length in lethal and non-lethal doses of the auxins, it is easy to infer that the pollen tubes in higher concentrations showed a far more significant increase in length (54—60 % in 10.0 and 100.0 mg./l. IAA; 24—100 % in 10.0 and 100.0 mg./l. IBA; 75—91 % in 10.0 and 100.0 mg./l. IPA) during 1—2 hours from sowing than that actually obtained in control or in lower and optimal concentrations of the test substances. This would seem to indicate that application of dilute concentrations of auxins continuously or stronger solutions for shorter intervals resulted in an increase in growth of the pollen tubes. This is true for certain other substances also under similar conditions of experimentation.

None of the vitamins had more than a slight promotive effect on the growth of the pollen tubes in the first 2 hours of sowing; obviously their inclusion did not appreciably affect the rate of elongation of the pollen tubes in the initial phase (Figures 4 B, 5 B). In doses of 10.0 and 100.0 mg./l. of PABA and AA, presumably because of the resistance of the pollen grains to high concentrations of the substances, the rate of growth was uniform and inhibition was noticed only after 12—24 hours. In lethal concentrations of inositol, on the other hand, the growth of the pollen tubes often and always decreased to a low level in the early hours of sowing. The 'peaks' in serial concentrations of PABA and AA were rather varying, but in inositol they were constantly observed at 6—8 hours.

The nature of response of the pollen tubes in the trace metals varied markedly with groups of substances and their concentration. The relative growth in all concentrations of H_3BO_3 extended up to 12 hours and response to the stimulant was evident even from the first hour of sowing (Figure 6 B). Pronounced growth was evident at 6—8 hours in non-lethal, at 5—6 hours in optimal and at 3—6 hours in lethal concentrations. In $CoCl_2$ and $LiCl$ maximum stimulation has been observed in lower concentrations (Figure 7 B). In non-lethal doses stimulation was entirely confined to the second phase and was preceded by a period of inactivation in the first phase; the peak was at 6—8 hours in $LiCl$ and at 6—12 in $CoCl_2$. The data would further indicate that whereas the rate of elongation in $CoCl_2$ extended up to 12 hours, in $LiCl$ no further elongation was observed after 8 hours. In concentrations of 10.0 and 100.0 mg./l. of both substances, inhibition was noted even in the early hours of sowing. With $MnSO_4$, $(NH_4)_2 MoO_4$, $ZnSO_4$ and $AuCl_3$ well marked



Figures 6—9. Three dimensional graphs showing the rate of germination of the pollen grains and growth of the pollen tubes in serial concentrations of H_3BO_3 , $CoCl_2$, $MnSO_4$ and $(NH_4)_2 MoO_4$ in relation to time. Figures 6 A, 7 A, 8 A, 9 A: Germination. Figures 6 B, 7 B, 8 B, 9 B: Pollen tube growth.

stimulation persisted up to 24 hours, especially in non-inhibitory concentrations. During the first hour in culture accelerated growth of the pollen tubes occurred in all concentrations (Figures 8 B, 9 B). The pollen grains treated with MnSO_4 then began rapid expansion with a peak at 12—24 hours. There was a progressively increased growth from the second hour onwards in $(\text{NH}_4)_2 \text{MoO}_4$ and AuCl_3 and from the third hour onwards in ZnSO_4 with peaks at 5—6, 8—12 and 5—12 hours respectively. As with other substances, higher concentrations resulted in progressively less increase and finally in complete inhibition of growth, evident at 2—3 hours from sowing in MnSO_4 , $(\text{NH}_4)_2 \text{MoO}_4$ and ZnSO_4 and at 24 hours in AuCl_3 .

Discussion

The results, when briefly reviewed, reveal the following:

Auxins and other micronutrients are capable of increasing the germination rate of pollen grains, probably also affecting the time at which germination starts, the magnitude of the effect varying in substances from pronounced stimulation to inhibition. The artificial test substances thus serve the purposes both of early stimulation of germination and more important, influence specifically the growth of the pollen tubes. This is significantly apparent in some cases as in IAA where the enhancement of the germination rate was not marked in the first hour, but the rate of pollen tube growth was seven-fold that in control. It is, therefore, reasonable to assume that the beginning of germination does not necessarily depend on the presence of some external stimulus, but its presence certainly affects and is intimately connected with the continued growth of the pollen tubes. An attempt to test this alternative was made by carrying out a slightly modified experiment in which the pollen grains were treated with the growth substances for 1 hour periods. Frequent observations of the cultures confirmed that the pollen grains were germinating to produce short tubes. The samples were subsequently washed thoroughly and carefully in water and further allowed to grow in sucrose. The results showed that pretreatment did not produce any more increase in length of the pollen tubes than that would have normally occurred when germinated and grown in sucrose alone. On the other hand, pollen grains germinated in sucrose and later transferred to stimulants showed spectacular increase in length of the pollen tubes. A check on the stimulation by test substances without sucrose was not possible, because sucrose or a carbohydrate is necessary for proper germination of the pollen grains, nutrition, osmotic control and

possibly other reasons (O'Kelley 1955, Raghavan and Baruah 1956 a, Visser 1955). In the stimulation of the pollen grains it is thus found that the artificial stimulants are not needed particularly until the pollen tubes are formed. Germination involves mechanism more than bulging of the pollen and protrusion of the tubes and it is apparent why the presence of a particularly active substance accelerates germination also, because its presence in many cases may determine to a large extent whether pollen grains will form visible tubes or not (Visser 1955).

Although evidence is clear that the concentration of the applied substance is the normal factor limiting pollen germination and pollen tube growth, the duration of treatment to produce the effect also seems to be equally responsible. The time of treatment thus determines how much substance gets into the pollen to produce an optimum effect. It is found that with increasing concentrations of the test substances in cultures, the time of treatment to produce the final effect was shortened, probably due to the shortening of the period required by the substances present in the external medium to reach an effective concentration in the pollen grains. When the effective concentration is reached, further increase produces no visible effect; on the other hand, if the pollen tubes are allowed to remain continuously in high concentrations of the solutions, they are considerably damaged. This phenomenon has also been demonstrated in our preliminary experiments. The pollen grains after being germinated in lethal concentrations for 1—2 hours or in non-lethal doses till inhibition was evident, were transferred to a basal medium of sucrose or one containing a ten-fold lower concentration of the substance; no growth in length of the pollen tubes was evident. However, while the simplest explanation for the inhibition appears to be an increased inhibitor level induced by the substances, this need not necessarily be the correct one. In certain instances of growth phenomena involving IAA, Tang and Bonner (1947) have attributed regulation and inhibition of growth due to the presence of IAA-destroying system (IAA oxidase). Lowering of growth rate in auxins has also been suggested as due to the overcrowding of the growth centres in the cells by the auxin molecules, so that they hinder each other sterically and the correct combination between the auxin molecule and the receptor cannot be obtained (Bentley and Housley 1953). While these may be true for IAA and other substances acting under certain conditions, further work will be necessary to determine their specific reactions in germinating pollen grains.

With the results at hand it has not been possible to determine the ultimate function of the substances within the pollen grains which manifests in increased germination of the pollen grains and growth of the pollen tubes.

According to Addicott (1943), they may function in the same ways as in other parts of the plant, namely, IAA and other hormones for cell elongation and water soluble vitamins for the enzymatic systems in respiration. O'Kelley (1957) has found that boron exercises a stimulatory effect on oxygen uptake and sugar absorption, besides having a specific role in pollen tube growth. Pollen tube growth under boron stimulation may also involve synthesis of the pectic materials of the growing pollen tubes or the synthesis of proteins. These observations need further corroboration by detailed work; the ease of germination of pollen grains of arecanut and the marked and differential stimulation they exhibit make it an easy material for studies associated with the ultimate function of growth substances in growing pollen tubes and the mechanism of pollen tube growth under hormonal stimulation. The results of such studies will be published elsewhere.

Summary

Experiments on the stimulation of pollen grains of *Areca catechu* Linn. by certain auxins, vitamins and trace elements have been extended to studies on the effects of time factor on the rate of germination of pollen grains and pollen tube growth. Addition of IAA, IBA, NAA, H_3BO_3 , $MnSO_4$, $(NH_4)_2 MoO_4$, $ZnSO_4$ and $AuCl_3$ in non-lethal concentrations to a basal medium of sucrose has been found to specifically increase the germination of the pollen grains and growth in length of the pollen tubes even from the first hour of sowing; the time required to reach the maximum effect is also reduced considerably. On the other hand, effects of PABA, AA and inositol on the pollen grains appear to be quite different and no initiation of response was found in the first hour; the period of activity of the pollen grains in these substances also persisted till 24 hours. In IPA there was an initial period of inhibition. With all substances inhibition in lethal concentrations was evident 2—3 hours from sowing. It is concluded that stimulants of the nature of auxins, vitamins and trace elements have a specific role to play in the growth of the pollen tubes; they also accelerate the germination of pollen, probably affecting the time at which germination starts. Evidence is interpreted to mean that the growth substances penetrate the pollen grains and induce positive responses, till an equilibrium concentration is reached; further penetration inhibits growth rate and damages the pollen tubes.

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Glucose Assimilation in Normal and Manganese Deficient *Chlorella* Cells

By

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Material and Methods

The alga, *Chlorella vulgaris* 211/11 h Beijerinck, was initially grown autotrophically in a medium which was composed as follows:

KNO ₃	0.81 gms.	CaCl ₂ · 6H ₂ O	0.02 gms.
NaCl	0.47 „	FeSO ₄ · 7H ₂ O	0.001 „
Na ₂ HPO ₄ · 2H ₂ O	0.18 „	ZnSO ₄ · 7H ₂ O	0.001 „
NaH ₂ PO ₄ · 2H ₂ O	0.47 „	MnCl ₂ · 4H ₂ O	0.0002 „
MgSO ₄ · 7H ₂ O	0.25 „		

in 1 liter of twice distilled water. The pH of the medium was 6.3.

All cultures were grown at room temperature in pyrex glass flasks and the autotrophic and mixotrophic cultures were exposed to a light intensity of 4,000 lux. Both normal and manganese deficient cultures were grown. No manganese was added to the manganese deficient cultures. After 10 days autotrophic growth 0.5 % glucose was added to the cultures and the cultures were divided so that normal and manganese deficient cells were grown mixo- and heterotrophically.

The cultures were examined analytically immediately after the addition of glucose and then at intervals over 4 days.

Dry weight was determined in the normal way by drying the algal cells at 105°C, after filtering through weighed sintered glass crucibles containing fine asbestos.

Chlorophyll (a and b) was determined by a spectrophotometric method in methanol solution at wave length of 600 mμ by using the formula of Smith and Benitez (6) and Comar and Zscheile (1) for the determination of the chlorophylls in ethyl ether

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Table 1. *Ratio of carbohydrates in cells to chlorophyll content.*

Cultures	Day 0	Day 1	Day 2	Day 4
Mixotrophic + Mn	2.27	4.65	6.6	5.3
Mixotrophic — Mn	4.1	9.7	2.85	1.65
Heterotrophic + Mn	2.27	6.0	6.6	5.6
Heterotrophic — Mn	4.1	6.1	2.25	1.04

solution and a calibration curve linking the spectrophotometric readings for chlorophyll in methanol solution and in ethyl ether solution was drawn. Chlorophyll was transferred from the methanol solution to ethyl ether solution by washing with sodium chloride solution and water and then drying over anhydrous sodium sulphate (Mackinney 3).

Two separate determinations of total carbohydrate, the total carbohydrates of the medium alone and of the algal cells alone were made. The method for the determination of carbohydrate has previously been described (2).

Results

Figure 1 shows the growth curves of the mixo- and heterotrophically grown cells. All the cultures showed rapid rise and decline in dry weight, associated with the rapid assimilation of glucose by the glucose starved cells which is subsequently respired. The curves for the chlorophyll content (Figure 2) show a general increase in chlorophyll content. The chlorophyll contents of the heterotrophic cultures were finally slightly higher than the chlorophyll contents of the mixotrophic cultures due probably to the chlorophyll destroying action of light. Figures 3 and 4 show the level of total carbohydrates in the medium and in the cells. While the carbohydrate content of the medium decreases in all cultures at approximately the same rate and reaches the same low level, the carbohydrate contents of the manganese deficient cells, both mixo- and heterotrophic, were much lower than the carbohydrate contents of the normal cells. So that although the absorption of glucose is approximately the same for all cultures it is metabolized more rapidly in the manganese deficient cultures. Table 1 of the ratio of carbohydrate in the algal cells to their chlorophyll content shows these results in greater relief, particularly on the fourth day.

Discussion

The growth curve (Figure 1) shows that four days after the addition of glucose all the cultures have similar dry weights. Although some of the glucose undoubtedly is transformed into cell substance the rate of this trans-

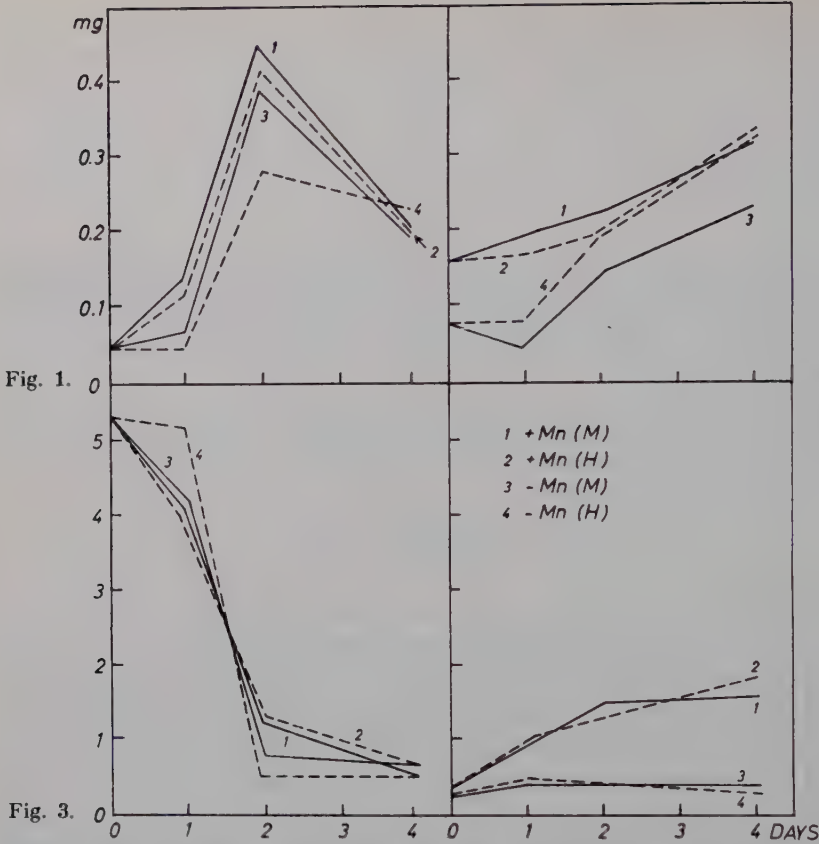


Figure 1. Growth curves of Mixotrophic (M) and Heterotrophic (H) cultures of *C. vulgaris* with and without the addition of manganese.

Figure 2. Chlorophyll contents of cells from heterotrophic and mixotrophic cultures of *C. vulgaris*.

Figure 3. Carbohydrate contents of media of heterotrophic and mixotrophic cultures of *C. vulgaris*.

Figure 4. Carbohydrate contents of cells from heterotrophic and mixotrophic cultures of *C. vulgaris*.

formation appears to be the same for all cultures. Therefore it must be argued that this data indicates indirectly that the respiration rate in manganese deficient *Chlorella* cells grown under these conditions is higher than that of normal cells.

Apparently these results are contrary to those of Pirson, Tichy, and Wilhelm (5) who found that the respiratory quotient of manganese deficient *Ankistrodesmus* cells was not appreciably different from that of normal cells.

It is possible, however, that this was due to the different conditions under which the cultures were grown. Pirson (4) states that, in light, manganese deficient unicellular algae cease glucose consumption. Nevertheless, it seems that under some conditions, as the present work shows, cultures of manganese deficient unicellular algae in light can metabolize glucose.

Summary

Normal and manganese deficient *Chlorella* cells were grown autotrophically, then glucose was added to the medium and the cells grown mixo- and heterotrophically. The total carbohydrates in the medium were metabolized at approximately the same rates and reached the same low levels in all cultures. But the total carbohydrates in the normal cells was much higher than the total carbohydrates in the manganese deficient cells. It could be inferred from this that the manganese deficient cells, grown under these conditions, had a higher respiration rate than the normal cells.

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Rendering the Germination of Light-Insensitive Lettuce Seeds Sensitive to Light

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It is well known that nonionizing radiation in the general region of the far red and near infrared increases frequency of X-ray-induced chromosomal aberrations in plants (11) and animals (4). It has been shown that this action of the nonionizing radiation has two features characteristic of the far-red-light effect on photocontrol of seed germination, flowering, and morphogenic effects on dark-grown seedlings: First, a common action spectrum in the far red (13) and, second, an apparent mutual antagonism between the effect of such far-red light and the effect of red light (7, 8).

Interaction of far-red light and X-rays in production of chromatid aberrations has been described as a potentiation of X-ray effects by far-red light. Considering the findings of Moh and Withrow, we may now alternatively describe the interaction as an X-ray induction of red, far-red photosensitivity. The work reported here was performed to determine whether an analogous interaction between far-red light and X-rays could be shown for lettuce seed germination, one of the classical systems for the study of red, far-red interactions. If an analogous interaction does occur, then we might expect to find that X-rays would induce photosensitivity to red and far-red light in lettuce seeds whose germination was otherwise unaffected by red and far-red light. For this purpose, as well as other reasons to be discussed, experiments were performed with a variety of lettuce seeds normally light insensitive at the

¹ Operated by Union Carbide Corporation for the United States Atomic Energy Commission.

temperatures used. This work shows an X-ray-induced sensitivity to red and far-red light in lettuce seed germination that is analogous to the X-ray-induced sensitivity to red and far-red light in the chromatid aberration system. The capacity of injurious treatments (other than X irradiation) to similarly induce photosensitivity to red and far-red light in the germination of such "light-insensitive" lettuce seeds was also studied.

Materials and Methods

Unless otherwise indicated, seeds of *Lactuca sativa*, var. New York, were used. Seeds were placed upon a filter paper moistened with 3.5 ml. of distilled water or solutions as indicated at pH 5.5 in covered 9-cm. petri dishes. The experiment reported in Table 1, however, was performed with 5.5-cm. dishes (containing a piece of filter paper moistened with 1 ml. of distilled water). These dishes were arranged in a light-tight container on a rotating turntable under the X-ray beam so that all dishes received equal X-ray doses. About 140 seeds were placed in each 9-cm. dish (100 per 5.5-cm. dish). The criterion of germination was visual detection of radicle protrusion. Data from replicate dishes were pooled for statistical analysis only when no significant differences were found according to the $R \times 2$ Table (10).

Seeds designated "frozen" were transferred, after various imbibition times, to a porcelain evaporating dish and covered to a depth of 2 cm. with liquid nitrogen, which evaporated within a few minutes at room temperature.

Light-treatments were given as indicated. Source of red light (590—680 $m\mu$) was a 150-watt incandescent bulb shining through a running-water filter, 2 cm. of 50 g./l. of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in 0.38 % H_2SO_4 , and one sheet of No. 67 gelatin filter (Brigham Gelatine Co., Randolph, Vt.). The far-red (710—1150 $m\mu$) source was a 150-watt incandescent bulb shining through a running-water filter and a Corning glass filter No. 7-69. The running-water filters consisted of 2.5 cm. of water bounded by 6-mm. sheets of Plexiglas (Rohm & Haas, Knoxville, Tenn.). The light sources were in wooden boxes that were light-tight except for the filter systems. The transmission spectra of the filters are shown in Figure 1. The Plexiglas showed no differential absorption from 400—1300 $m\mu$.

The red and far-red sources delivered 4.9 and 93 ergs/mm.² sec., respectively. White light was 20-ft.-c. incandescent lighting.

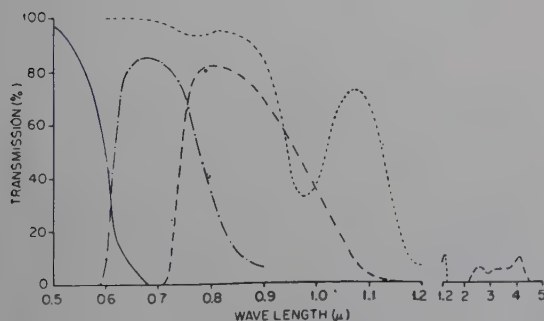


Figure 1. Transmission of filters.
 — · — · —, Brigham gelatin No. 67.
 —, copper sulfate. — — —, Corning glass No. 7-69. - - - - -, water.

Results

Neither the rate nor the extent of germination of untreated New York lettuce seeds was significantly affected by light conditions at 21—22°C (Figures 2, 3). However, when such seeds were X irradiated during imbibition with doses that only slightly affected their subsequent germination under white light, their germination under far-red light was greatly inhibited. The effect of far-red light was to reduce the total extent, not merely the rate, of germination. Seeds that had been given 1000 r./min. during the first 45 minutes of imbibition and then 24 hours of far-red treatment never attained full germination capacity even if subsequently transferred to white light for several days.

When New York seeds were X irradiated in darkness and remained in darkness interrupted only by exposure to red and/or far-red light, it was seen that red light could promote and far-red light could inhibit germination in darkness (Table 1). This conclusion is based on the partition of χ^2 given under Table 1, which (like the partitions of χ^2 under Tables 2, 4, and 5) was designed to assay the significance of (a) the germination-inhibiting effect of far-red light after red light-treatment when compared with the effect of no light exposure after red light-treatment, and (b) the germination-promoting effect of red light after far-red treatment as compared with that of no light

Table 1. *Photoreversal of light effects on X-irradiated New York lettuce seeds* (kr./min. 250-kvp during first 45 minutes in darkness). Light-treatment 2 hr. begun immediately after X-irradiation.

Light-treatment	% Germination (24 hr., 22°C)	
None	16	
Red	29	
Far-red	13	
Red+far-red	9	
Far-red+red	33	
$\chi^2 = 26$	d.f. = 4	$p < 0.0001$
$\chi^2_1 = 12.4$	d.f. = 1	$p = 0.0004$
$\chi^2_2 = 12.0$	d.f. = 1	$p = 0.0005$

χ^2 was computed for four degrees of freedom by the method of the R. 2 Table (10). This value of χ^2 could be partitioned into four orthogonal components each with one degree of freedom (5). χ^2_1 and χ^2_2 are two such orthogonal components, referring to the comparisons: red vs. red+far-red, and far-red vs. far-red+red respectively.

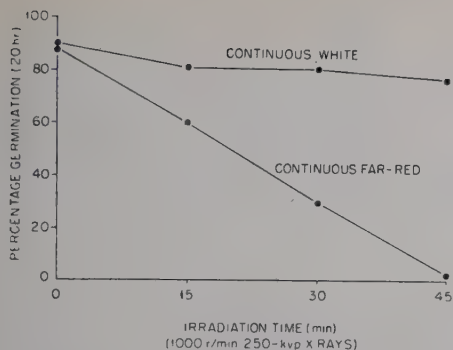


Fig. 2.

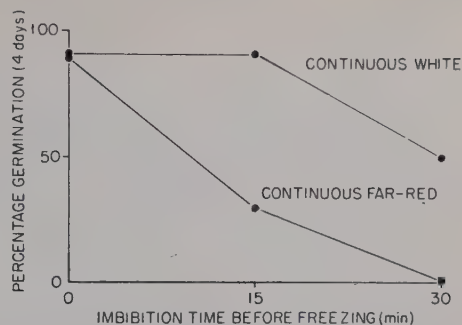


Fig. 3.

Figure 2. Effects of X irradiation and subsequent light conditions on germination of New York lettuce seeds at 22°C. Imbibition was begun at 0 time.

Figure 3. Effects of freezing in liquid nitrogen and subsequent light conditions on germination of New York lettuce seeds at 22°C.

exposure after far-red treatment. These effects of light on X-irradiated New York seeds are the same as the effects of red and far-red light on germination of varieties of lettuce seeds normally light sensitive (1).

To determine to what extent the photosensitivity induced in New York seeds was peculiar to X-ray damage, seeds were also subjected to other injurious treatments. It is well known that dry seeds are not generally susceptible to freezing damage, but that wet tissues can be damaged or killed by freezing (6). Thus freezing damage to seeds can be regulated by freezing at varying times while the water content of the seeds is increasing. When seeds

Table 2. Photoreversal of light effects on New York seeds frozen after 15 minutes' imbibition under white light. Light-treatment 2 hr. immediately after freezing treatment.

Light-treatment	% Germination (4 days, 22°C)
None	66
Red	73
Far-red	52
Red+far-red	57
Far-red+red	69

$\chi^2 = 16.5$	d.f. = 4	p = 0.0024
$\chi^2_1 = 6.54$	d.f. = 1	p = 0.0106
$\chi^2_2 = 8.63$	d.f. = 1	p = 0.0033

χ^2 , χ^2_1 , χ^2_2 as in Table 1.

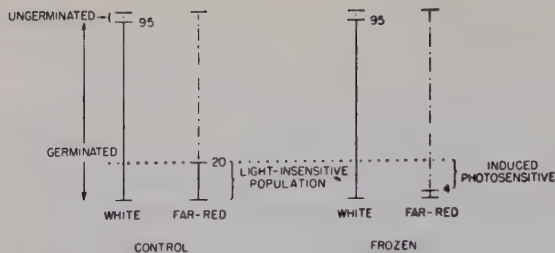


Figure 4. Rendering the light-insensitive population of Grand Rapids seeds sensitive to far-red treatment by freezing after 10 minutes' imbibition. Lengths of vertical lines and numbers indicate percentage of total population for each of the four treatments.

were suitably damaged by freezing, their subsequent germination was reduced under far-red light far more than under white (Figure 3). The photosensitivity induced by freezing damage seemed to be the familiar red, far-red photoreversible type (Table 2).

New York seeds were also germinated in the presence of either of two chemical inhibitors of germination: mannitol and 2,4-dinitrophenol. The general growth- and germination-inhibiting properties of these chemicals have been attributed to osmotic effects and the uncoupling of oxidation from phosphorylation, respectively. Table 3 shows that, in the presence of either of these inhibitors, germination was more reduced under far-red than under white light. Just as with the photosensitivity induced by X irradiation or freezing, the photosensitivity induced by mannitol or dinitrophenol seemed to be the red, far-red photoreversible type (Tables 4, 5).

Experiments similar to those just described were also done with Grand Rapids, a variety of lettuce seed that is normally photosensitive under these experimental conditions. Figure 4 illustrates the results of one experiment. Untreated Grand Rapids seeds gave 20 % germination under far-red and 95 % under white light. When seeds were suitably damaged by freezing, germination under far-red was reduced to 4 %, whereas under white light it was still 95 %. We may consider that the unfrozen control seeds used here consisted of two populations: one in which all seeds were sensitive to far-red (75 %) and one in which none of the seeds were sensitive to far-red (20 %). This 20 % of the untreated seeds thus behave qualitatively like our untreated New York seeds. When the light-insensitive population of Grand Rapids seeds

Table 3. Effects of chemical inhibitors and light on germination of New York seeds. Light-treatments were given continuously.

Supplement	Percentage germination (24 hr.)	
	White light	Far-red light
None	92	85
0.1 M mannitol	85	7
10 ⁻⁴ M 2,4-dinitrophenol	77	17

Table 4. *Photoreversal of light effects on New York seeds in 0.2 M mannitol. Light-treatment 2 hr. from beginning of imbibition.*

Light-treatment	% Germination (24 hr., 22°C)
None	4.2
Red	9.6
Far-red	3.2
Red+far-red	4.5
Far-red+red	11.6
$\chi^2 = 21$	d.f.=4 p=0.0003
$\chi^2_1 = 4.87$	d.f.=1 p=0.0273
$\chi^2_2 = 13.6$	d.f.=1 p=0.0002

χ^2 , χ^2_1 , χ^2_2 as in Table 1.

was damaged by freezing, however, 16 of every 20 seeds were rendered sensitive to far-red light. Consequently, had we been able to sort out and use only the light-insensitive population of Grand Rapids seeds, the results would have been similar to those obtained with New York seeds. From the foregoing argument, it is clear that rendering the germination of light-insensitive varieties of lettuce seeds sensitive to light is a special case of the more general modification of photosensitivity of light-sensitive varieties of lettuce seed. Much data has accumulated showing that chemical treatments can indeed modify the photosensitivity of Grand Rapids seeds (3, 12). Thus, the experiments performed with New York seeds could theoretically have been done with Grand Rapids seeds. The conclusions would have been realized with more difficulty, however, since it would have been much harder to detect significant differences working downward from a 20 % germination than from 90 %.

Table 5. *Photoreversal of light effects on New York seeds in 10^{-4} M 2,4-dinitrophenol. Light-treatment 2 hr. from beginning of imbibition.*

Light-treatment	% Germination (20 1/2 hr., 22°C)
None	29
Red	47
Far-red	20
Red+far-red	29
Far-red+red	55
$\chi^2 = 30$	d.f.=4 p<0.0001
$\chi^2_1 = 8.9$	d.f.=1 p=0.0029
$\chi^2_2 = 17.9$	d.f.=1 p<0.0001

χ^2 , χ^2_1 , χ^2_2 as in Table 1.

Discussion

In none of the experiments with New York seeds did far-red treatment alone significantly inhibit germination. Thus the interaction between X-rays and far-red treatment in inhibiting germination of lettuce seeds was analogous to the interaction of these two radiations in the production of chromatid aberrations (13). The analogous interactions of far-red and X radiation in production of chromatid aberrations on the one hand, and on the inhibition of lettuce seed germination on the other, do not necessarily imply common mechanisms of interaction for these two different systems. Similar interactions on lettuce seed germination were also found when other injurious treatments were substituted for X irradiation. Consequently, the induction of red, far-red photosensitivity in the production of chromatid aberrations might also be detected, in the absence of X irradiation, under other conditions detrimental to the maintenance of chromosomal integrity.

The results reported here also have implications for other studies of photo-control of germination. It has been reported that coumarin (9) and high temperatures (1, 2) were capable of inducing photosensitivity in varieties of lettuce seeds normally insensitive to light. The present results emphasize that a wide variety of injurious physical and chemical treatments may unmask latent photosensitivity. Since lettuce is a cold-weather crop, high-temperature treatment can be considered as merely one of the many kinds of injurious treatments that can induce photosensitivity in lettuce seed germination. Such considerations of the effects of high-temperature treatment may provide a reasonable alternative to the explanation that high-temperature effects on the light requirements of the germination of Grand Rapids seeds result from a simple thermal conversion of the postulated photoreceptive pigment from the far-red-absorbing to the red-absorbing form (1).

Perhaps it should be mentioned here that the criterion of red, far-red photosensitivity for germination of New York seeds was the effectiveness of red and far-red light after far-red and red treatments, respectively. In Tables 1, 4, and 5, there is little or no significant effect of a far-red treatment only, compared with dark controls. In Table 2 there is little or no effect of red treatment only, compared with dark controls. These different behaviors of New York seeds damaged in different ways correspond to those of Grand Rapids seeds, in which germination in darkness may vary from the high level of those treated with red light to the low level of those treated with far-red light. But in each case, red or far-red light is effective after treatment with far-red or red light, respectively.

Finally, we may consider Grand Rapids seeds to be similar to New York seeds except for some inherent, perhaps genetic, damage, which results in their greater photosensitivity.

Summary

New York lettuce seeds germinate equally well irrespective of light conditions at 21—22°C. When such seeds were damaged by X irradiation, freezing during imbibition, or germination in mannitol or 2,4-dinitrophenol, their germination was more reduced under far-red than under white light. The photosensitivity induced by each of the four agents appeared to be of the well-known red, far-red photoreversible type present in varieties of lettuce seed normally photosensitive. These findings have possible implications both for studies of photocontrol of seed germination and for studies of interactions between far-red and X radiation in the production of chromatid aberrations.

The author gratefully acknowledges the able participation of Mrs. Helen J. Luipold in portions of this work, the advice on statistical procedures given by Dr. M. A. Kastenbaum, and the assistance in calibrating light sources given by Dr. John Jagger, all of the Oak Ridge National Laboratory.

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Investigations on a Cofactor for the IAA Oxidase of Peas

By

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Introduction

It has been shown that the indoleacetic acid (IAA) destroying enzyme referred to as IAA oxidase has a requirement for an organic cofactor of low molecular weight (6,8). This requirement may be satisfied by a number of compounds including substituted phenols (2) but the identity of the naturally occurring substance or substances which may function as a cofactor has received little attention. A recent report (3) describes the isolation from pineapple of esters of ferulic and p-coumaric acid which can act as cofactors in the enzymatic oxidation of IAA. Neither of these can, however, be the substance active in pea preparations for reasons of solubility and lability described below.

This report describes methods of partial purification of an organic IAA oxidase cofactor from peas, as well as observations on an enzyme system constructed from pea breis. Complete isolation and final identification of the factor have not been achieved, but we believe that sufficient interesting information has been obtained to warrant publication at this time.

Materials and Methods

All enzyme preparations and organic cofactor preparations were obtained from Alaska peas grown at 27° in the dark. For early experiments, roots of 3-day-old peas were used, while for later experiments the aerial portions of 7- or 8-day-old plants were used. In the latter case enzyme preparations were made either from the stems

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from which apical buds had been removed or from the buds themselves. The frozen tissues were ground in a mortar with water, the resulting brei squeezed through cheesecloth, and the filtrate dialyzed for 24—48 hours against 25 volumes of 0.01 *M* phosphate buffer, pH 6.1, with two or three changes of dialyzing medium during the first 24-hour period. This procedure was usually adequate for the preparation of enzyme solutions with negligible endogenous activity under the experimental conditions employed. Since preparations varied widely in activity, they were assayed under standard conditions and used in subsequent experiments in amounts such that reaction rate was independent of enzyme concentration.

The cofactor preparations were made from apical buds by a method adapted from that of Cruikshank (1) for extraction of polyphenols. The buds were first extracted in a Waring blender with peroxide-free ether (Mallinckrodt) on a 1 : 3 (wt./vol.) basis. This extraction was repeated until the extract had become colorless. The ether extracts, which contained negligible amounts of cofactor, were discarded. The solid residue was then extracted in the blender with 80 % ethanol on a 1 : 4 (original tissue wt./vol.) basis. After filtration of the slurry through a Büchner funnel, the solid residue was washed with one volume of alcohol, and then re-extracted with four volumes of alcohol. Filtrates from the two alcohol extractions and washings were pooled and reduced to a small volume in a flash evaporator at 30°. The dark yellow solution was then used as a crude cofactor preparation.

In early experiments, cofactor preparations were obtained directly from aqueous extracts, being freed of protein either by boiling or by dialysis. Since the alcohol extraction procedure proved to be more convenient, it was used in the bulk of the experiments.

The assay used was that developed by Tang and Bonner (7), which depends on a colorimetric determination of residual IAA in the presence of $\text{FeCl}_3 \cdot \text{H}_2\text{SO}_4$ reagent. Enzymatic destruction of IAA was carried out at 26°C. in a Dubnoff shaker.

Results

Figure 1 shows the effect of addition of a crude boiled aqueous extract from the terminal 2-millimeter segment of the root to an enzyme from a segment 3 millimeters long just back of this. Initial experiments performed with roots indicated that the factor was localized in the younger tissues. The bulk of the extractions was made from terminal bud tissue since, as with roots, the factor is localized in the younger portions of the stem.

Figure 2 shows a dependence of enzyme activity on cofactor level, the cofactor being obtained by dialysis of an aqueous extract of terminal bud tissue. Although no measurable activity was found in the absence of cofactor, a parallel experiment with double the amount of enzyme showed a slow destruction of IAA. It should be noted that no enzyme preparations were found to be completely free of activity, although endogenous activity was always very low.

The effect of manganese in a system containing crude dialysate is shown in Figure 3. At suboptimal concentrations of Mn^{++} there is a pronounced lag

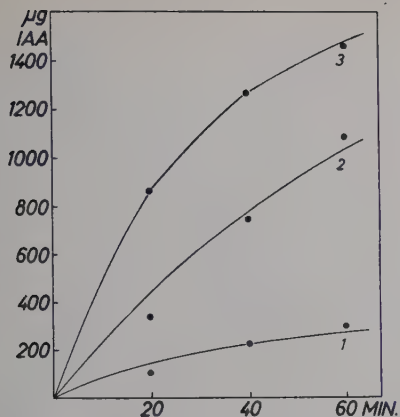


Fig. 1.

Figure 1. *Effect of a boiled aqueous extract from pea roots on the activity of an IAA oxidase preparation from the same source.* Contents of complete reaction mixture: 1 ml. enzyme preparation, 1.25 ml. boiled extract, 0.5 ml. 0.1 *M* phosphate buffer, pH 6.1, 1.25 ml. 4×10^{-4} *M* IAA, 1 ml. 2.5×10^{-4} *MnCl*₂. Total volume, 5.0 ml. On the ordinate µg IAA destroyed per mg. protein N. 1=Enzyme alone; 2=Enzyme+extract; 3=Enzyme+extract+*Mn*⁺⁺, 5×10^{-5} *M*.

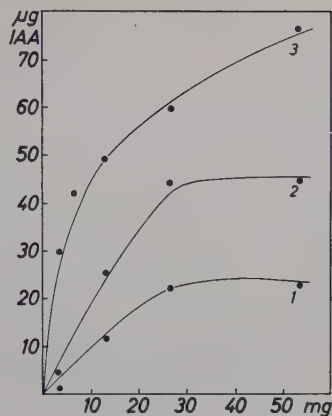


Fig. 2.

Figure 2. *Dependence of rate of IAA destruction by a dialyzed enzyme preparation from pea buds on concentration of the added dialysate (mg. f.w./ml.).* Contents of complete reaction mixture: 0.4 ml. enzyme; 0.15 ml. 1×10^{-4} *M* *MnCl*₂; 0.75 ml. 1×10^{-3} *M* IAA; 0.9 ml. 0.033 *M* phosphate buffer, pH 6.1; 0.8 ml. dialysate. Total volume, 3.0 ml. On the ordinate µg IAA destroyed per reaction mixture. 1=30 min. —*Mn*; 2=60 min. —*Mn*; 3=60 min. +*Mn*⁺⁺, 5×10^{-5} *M*.

period which falls off rapidly to a negligible level, the concentration at which this level is reached being approximately optimal. Similar experiments extending the range to 1×10^{-3} *M* *Mn*⁺⁺ show a further marked decrease in reaction rate with increase in manganese concentration, with no increase in lag period.

Figure 4 illustrates the effect of pH on the activity of enzyme preparations supplemented either with 2,4-dichlorophenol (DCP) or the natural cofactor. In agreement with previous studies (5, 7) the optimum is about 5.5, both for DCP and the naturally occurring substance. The time relations for oxidation with the two substances differ however (Figures 5 and 6). No lag period was observed throughout the pH range employed where a crude extract was used, while with DCP a lag period was observed which was shortest at the optimal hydrogen ion concentration.

Where cofactor preparations were made by extraction of tissue with 80 % alcohol, the final extract formed a heavy white precipitate on standing over-

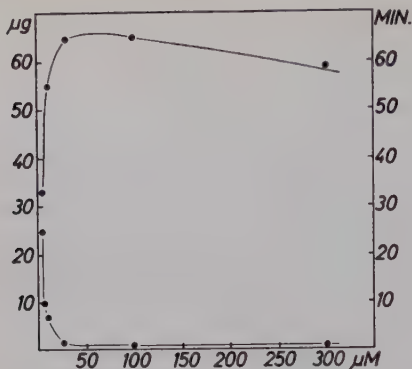


Fig. 3.

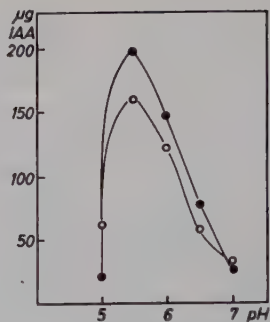


Fig. 4.

Figure 3. *Effect of manganese concentration (μM) on the rate of destruction of IAA by a dialyzed enzyme preparation from pea buds and on length of lag period.* Contents of reaction mixture: 0.15 ml. enzyme; 0.3 ml. MnCl_2 solution; 0.6 ml. 1×10^{-3} M IAA; 0.45 ml. 0.067 M phosphate buffer, pH 6.1; 1.5 ml. 200 mg. f.w./ml. dialyate. Total volume, 3.0 ml. On the left ordinate (upper curve) μg IAA destroyed/reaction mixture in 30 min.; on the right ordinate (lower curve) length of lag period in minutes.

Figure 4. *Effect of hydrogen ion concentration on rate of destruction of IAA by a dialyzed enzyme preparation from pea buds.* Contents of reaction mixture: 0.5 ml. enzyme; 0.3 ml. 1×10^{-3} M MnCl_2 ; 1.2 ml. 1.5×10^{-4} M DCP or 200 mg. f.w./ml. dialyate; 0.6 ml. 1×10^{-3} M IAA; 0.4 ml. 0.075 M phosphate buffer. Total volume, 3 ml. On the ordinate μg IAA destroyed/reaction mixture in 20 min. —●— DCP 6×10^{-5} M; —○— natural cofactor 80 mg. f.w./ml.

night in the refrigerator. With removal of this precipitate by centrifugation, the extract had apparently lost activity, which could be restored by addition of the precipitate fraction. The dependence of the reaction rate on a system containing varying levels of this fraction is shown in Figure 7. In Figure 8 are presented data showing the dependence of the reaction rate on the concentration of the supernate fraction at a constant level of precipitate fraction. The optimum appears to be further to the right in the system containing the higher concentration of precipitate.

That the effect of the precipitate fraction is to shorten the lag period is clear from Figure 9. No conclusions as to effect on final rate can be drawn from this experiment because a saturating level of supernate was employed.

The relationship of reaction rate to supernate level is shown in Figure 10. Here there is an increase in final rate with increased concentration of supernate, up to the point at which the supernate becomes saturating. From Figure 10 it is probable that the role of the precipitate fraction does not in-

Figure 5. Time course of IAA destruction at various hydrogen ion concentrations by an enzymatic system containing the naturally occurring cofactor. Reaction mixture composition given in Figure 4. On the ordinate μg IAA destroyed/reaction mixture.

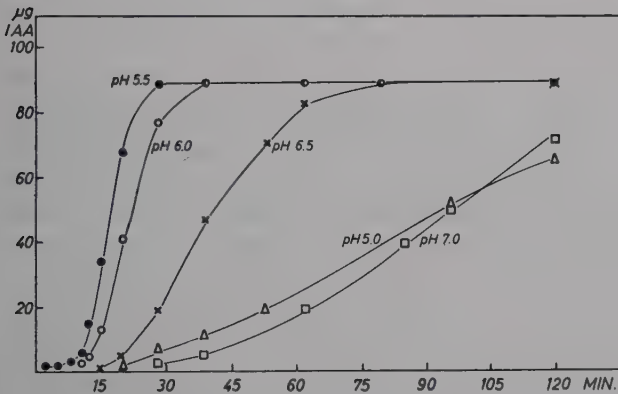
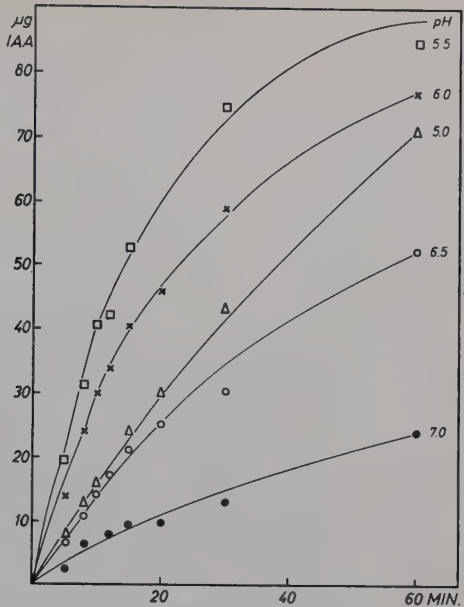


Fig. 6.

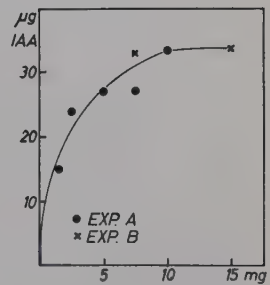


Fig. 7.

Figure 6. Time course of IAA destruction at various hydrogen ion concentrations by an enzymatic system containing 2,4-dichlorophenol as cofactor. Reaction mixture composition given in Figure 4. At equilibrium 30 $\mu\text{g}/\text{ml}$. IAA had been destroyed. On the ordinate μg IAA destroyed/reaction mixture.

Figure 7. Effect of concentration of precipitate fraction (fraction B, mg. f.w./ml.) on activity of an enzymatic system composed of enzyme from pea stems in combination with a supernate fraction (fraction A) derived from an alcoholic extract of pea buds. Contents of reaction mixture: 0.3 ml. enzyme; 0.06 ml. supernatant (fraction A), 750 mg. f.w./ml.; 0.3 ml. $5 \times 10^{-4} M$ MnCl_2 ; 0.6 ml. $1 \times 10^{-3} M$ IAA; 0.4 ml. 0.075 M phosphate buffer, pH 5.5; 0.06 ml. suspension of fraction B; 1.28 ml. water. Total volume 3 ml. Note: No reaction occurs in mixtures lacking ppt fraction. On the ordinate μg IAA destroyed/reaction mixture in 30 min. ● Expt. A × Expt. B.

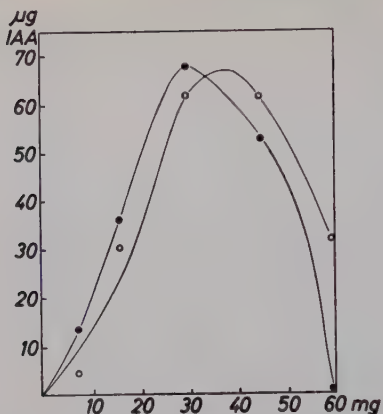


Fig. 8.

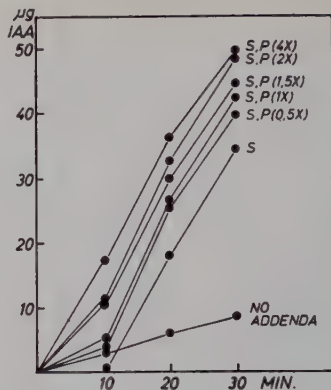


Fig. 9

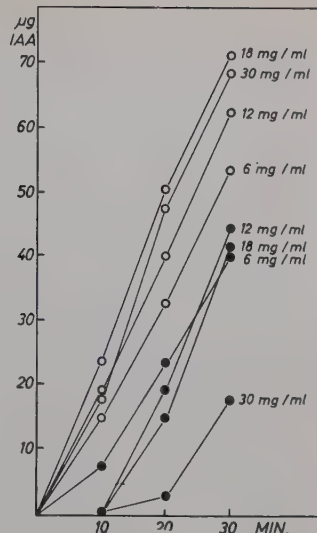
Figure 8. *Effect of concentration of supernate fraction (fraction A, mg. f.w./ml.) on activity of an enzymatic system composed of enzyme from pea stems in combination with a ppt. fraction derived from an alcoholic extract of pea buds.* Contents of reaction mixture as in Figure 7 except that concentration of supernate component is varied and that of ppt component fixed. (—●—), final concentration of B, 15 mg./ml.; (—○—), final concentration of B, 30 mg./ml. On the ordinate μg IAA destroyed/reaction mixture in 30 min.

Figure 9. *Time course of destruction of IAA in a system composed of enzyme and supernatant alone, and in a system containing various levels of precipitate in addition.* Contents of reaction mixture: 1.8 ml. enzyme; 0.16 ml. supernate, 2500 mg. f.w./ml.; 0.04 ml. precipitate suspension; 0.3 ml. 5×10^{-4} M MnCl_2 ; 0.6 ml. 1×10^{-3} M IAA; 0.1 ml. 0.3 M phosphate buffer, pH 5.5. Total volume 3 ml. Figures to the right of the curves represent the amounts of suspension relative to supernate on a mg. f.w. basis. S=supernate, P=precipitate. On the ordinate μg IAA destroyed/reaction mixture.

volve active participation in the reaction. This is deduced from the fact that addition of the precipitate fraction to a system containing a limiting amount of cofactor (supernate fraction) has no effect on the steady state rate (Figure 10, curves for 6 mg./ml.). Moreover, it has been found that with well-washed precipitates no reaction occurs in a system from which supernate is excluded at any level of precipitate up to 150 mg. f.w./ml. That the fraction does, however, affect the lag period is evident from both Figure 9 and 10.

The supernate fraction appears to contain an active cofactor and, according to Figure 10, this entity does not have a supra-optimal range. The descending limbs of the curves of Figure 8 are an expression of an increasing lag which is not compensated by the precipitate fraction at the levels employed. Experiments with higher precipitate levels, as well as time course

Figure 10. Time course of IAA destruction in a system containing various levels of supernate with and without precipitate fraction. Contents of complete reaction mixture: 1.6 ml. enzyme; 0.2 ml. supernate; 0.3 ml. 5×10^{-4} M MnCl_2 ; 0.2 ml. ppt. suspension, 450 mg. f.w./ml.; 0.6 ml. 1×10^{-3} M IAA; 0.1 ml. 0.3 M phosphate buffer, pH 5. (—●—) without precipitate fraction; (—○—), with precipitate fraction. On the ordinate μg IAA destroyed/reaction mixture.



experiments (Figure 10), indicate that no properties inhibitory to this enzyme system inhere in the unknown supernatant factor.

Attempts were made to isolate the active compound. Solubility tests showed it to be a highly polar substance, since it could not be extracted from aqueous solutions into diethyl ether or higher alcohols at any pH. It was retained by Dowex-1 chloride but not by Dowex-50. It was highly unstable at alkaline pH, but quite stable at acid pH when kept in the freezer. With storage in the refrigerator for 48 hours, a considerable portion of the activity was lost. It was stable to light. On Whatman #1 filter paper it migrated in tertiary butyl alcohol-water mixtures, being visible under ultra-violet light as a bluish area.

Several attempts were made to separate the substance in question on Dowex formate columns (prepared by alternately washing Dowex-1 chloride with large volumes of ammonium formate and formic acid) using the gradient elution technique. After application of a crude supernate fraction to the column followed by extensive washing with water, the column was eluted from a mixing flask filled initially with water which was connected to a bottle containing 20 % formic acid. Preliminary tests using small columns with successive elutions by aliquots of formic acid solutions of increasing concentration had indicated that the substance came off at 6–8 % acid. In one experiment a column 5 cm. long was eluted with a flow rate of 0.3 ml./min. cm. The eluate was collected in aliquots of about 3 ml. using a Reco fraction collector and each aliquot was brought to pH 5.5 with solid NaHCO_3 . Ninety fractions

were collected and every fifth fraction was tested for activity in a standard reaction mixture. Controls were run to check for destruction on the column — *i.e.*, fast elutions with 6—8 % formic acid of columns which had stood in the cold room the same length of time as the experimental columns were performed. Under these conditions there was no destruction of the active substance. Controls were also run to test for possible interference with the reaction by salt present after treatment with NaHCO_3 . Two fractions which had come off at 8—9 % acid showed weak but significant activity which, however, had disappeared within an hour after assay. This had been noted once before where assays were carried out the same day as the elution. When the fractions were assayed the following day no activity was detected. This suggests that after removal from the column in a somewhat purer form the substance rapidly deteriorates. Possibly a protective substance such as a sulfhydryl compound is involved in stabilization of the substance in the cruder mixtures.

The active fractions showed broad ultraviolet absorption peaks at 266 m μ and 317 m μ .

Discussion

The cofactor involved in this system is probably not that isolated by Gortner *et al.* from pineapple (3), since it has a marked sensitivity to alkali. Their compound was extracted and purified under conditions involving lengthy exposures to high concentrations of NaOH . The substance from peas also differs in solubility from the cofactors of pineapple, lupine and wheat, which are readily soluble in ether (3, 6, 8).

It is interesting to note that the pea cofactor exhibits no optimum concentration, in distinction to DCP (optimum in this system, $6 \times 10^{-5} M$), *o*-cresol, resorcinol, *p*-phenylenediamine, etc. (2). According to the Maclachlan-Waygood scheme, this would indicate a failure of the cofactor to react with manganese ion.

The most feasible interpretation of the lag period found with the supernate fraction is that there is present an inhibitor which is slowly broken down, the function of the precipitate fraction being to remove it, possibly by physical adsorption. With the method of extraction employed (80 % alcohol), it is possible that small polysaccharides in the extract could function in such a capacity. Simple tests indicate that no proteinaceous material is involved, nor can the precipitate effect be ascribed to metal ions. At any rate it would appear that the active component is contained in the supernate fraction and that the precipitate fraction plays a secondary role.

The relationship of length of lag period to manganese concentration is consistent with the Maclachlan-Waygood postulate of an initial build-up of manganic ions to a threshold concentration. A similar relationship holds where the organic cofactor used is DCP.

The occurrence of lag periods in the system containing DCP (Figure 6) is to be expected, since a sub-optimal level of Mn^{++} , $1 \times 10^{-4} M$, was used. The optimum for this system, if one exists, is greater than $1 \times 10^{-3} M$. However, the involvement of hydrogen ion level in the length of lag period is in marked contrast with the situation in a system containing natural cofactor, where there is no lag period at any pH. The effects in the DCP system cannot be ascribed solely to precipitation of insoluble Mn^{++} salts at higher pH's, since the lag period increases on the acid side as well.

In conclusion, the natural material of peas appears to differ from other substances of natural origin reported to behave as cofactors for an IAA oxidase in solubility properties and response to alkali. It also differs from all "artificial" cofactors with the exception of maleic hydrazide, in that it has no inhibitory properties and in the different pattern of destruction of IAA with time. Its properties are consistent with the conjecture that it is a substituted phenol, but confirmation of such a supposition must await isolation of the pure substance.

Summary

The purification of an indoleacetic acid oxidase cofactor from pea buds is described. Active extracts are obtained by 80 % ethanol extraction of terminal buds of etiolated peas previously extracted with diethyl ether. The alcoholic extracts, when stored in the cold, spontaneously separate into an inactive precipitate and an active supernatant fraction. The supernate shows no inhibitory properties, the steady state rate of IAA destruction increasing regularly with increased quantity added. The lag period may, however, be lengthened by unduly high concentrations of the supernatant. Addition of the precipitate to the supernatant then appears to increase activity by decreasing this lag period.

Attempts to fractionate the active material further by chromatography on Dowex-formate using a gradient elution technique resulted in loss of activity. Although the active material has not been successfully isolated, it is shown, on the basis of insolubility in ethyl ether and lability to alkali, not to be identical with IAA oxidase cofactors from pineapple, wheat or lupine.

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Uptake and Distribution of Radioactive Carbon from Labelled Substrates by Various Cellular Components of Spinach Leaves

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Introduction

Although considerable knowledge concerning the path of carbon in photosynthesis has been obtained by Calvin and co-workers (Bassham and Calvin 1957), little real progress has been made regarding our knowledge of the precise intracellular location of the many reactions of photosynthesis. The chloroplasts, which are responsible at least for the primary photochemical reactions generating the reducing power necessary for the conversion of free carbon dioxide to the level of carbohydrate, have been found to reduce pyridine nucleotides photochemically with a concomitant evolution of oxygen (Vishniac 1952). It has been suggested by other workers that, in addition to all the enzymes involved in the photosynthetic cycle, enzymes concerned with polysaccharide synthesis (Allen *et al.* 1955) and the respiratory activity of the Krebs cycle (Bassham *et al.* 1956) are also associated with the chloroplasts (*i.e.*, while the chloroplasts contain or are associated with a complement of these enzymes, the enzymes may also be present elsewhere in the cells).

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The chloroplast may thus be visualized as an entity which is capable not only of fixing and reducing carbon dioxide but also of metabolizing its reduction products via the Krebs cycle to the many organic acids and amino acids which are involved in or originate from Krebs cycle activity. It has been shown (Allen *et. al.* 1957) that isolated chloroplasts do fix carbon dioxide in the light, and that radioactive carbon from labelled carbon dioxide is in fact found in such compounds as phosphoglyceric acid (trace), hexose monophosphate, glucose and various amino acids. These results suggest that the chloroplast actually does contain the carbon dioxide fixation enzymes, together with enzymes converting phosphoglyceric acid (the first product of carbon dioxide fixation) into other substances. In fact, one may obtain a clear supernatant liquid rich in carboxydismutase by simply washing an intact chloroplast preparation with dilute buffer (Fuller 1955, Lyttleton 1958, Pon 1959). This idea is further strengthened by other results (Whatley 1956) showing that chloroplast fragments which have lost the capacity to fix carbon dioxide can have this ability restored to them by the addition of the clear supernatant liquid obtained after centrifugation of broken chloroplasts.

Bassham *et. al.* (1956) using *Chlorella*, have reported that radioactive carbon from labelled carbon dioxide is not found to any significant degree in glutamic acid (~5 min.) as long as the cells are in strong light, but is incorporated into this amino acid immediately after the light is switched off. This suggested that a small fraction of the total cellular complement of the respiratory enzymes of the Krebs cycle is associated with the chloroplast, as the incorporation of carbon-14 into glutamic acid upon turning off the light is so rapid that it must occur at the seat of the photochemical reception. Recent work (Holm-Hansen, in press) has shown that much more carbon from carbon dioxide is incorporated into glutamic acid when the cells were suspended in nutrient solution than when they were in distilled water, and hence there is some question as to the validity of the interpretation of this glutamic acid labelling data. Some attempts (Whatley 1956) have been made to find direct evidence for the presence of Krebs cycle enzymes in chloroplasts, but without success.

It is known that in plants and animals most of the Krebs cycle enzymes can be found in the mitochondrial fraction of the cells, whereas the enzymes associated with the glycolytic degradation of sugar are soluble in the cell cytoplasm (Neilands 1955). In view of the evidence demonstrating that some biochemical reactions, at least, are associated with distinct subcellular organelles, it is possible that the chloroplast may represent a body more or less specialized for the fixation and reduction of carbon dioxide, together with some simple transformations of the initial products of this fixation. The conversion of the products of carbon dioxide fixation into the wide variety of

metabolites found in the plant may, however, require the collaboration of many enzymatic systems found outside the chloroplast. Assuming that this is the case, the problem arises of the mechanism of transfer of molecules within the cell from one place to another, with the associated time delays, etc.

Although isolated chloroplasts have shown some of the reactions attributed to them in the intact cell, the rate at which carbon dioxide is fixed by these isolated particles is insufficient to account for the activity shown by the intact cell. Allen (1955) reported an activity of isolated chloroplasts corresponding to about $0.004 \mu\text{moles CO}_2 \text{ fixed/min. mg. chlorophyll}$. The rate of carbon dioxide uptake for the intact spinach leaf is not known to us, but the rate for *Chlorella* is $3\text{--}4 \mu\text{moles CO}_2 \text{ fixed/min. mg. chlorophyll}$ (Bassham, personal communication) (Hill 1955). The value given above for the rate shown by the isolated spinach chloroplasts is thus only about 0.1 % that of the maximum rate of intact *Chlorella*. On the other hand, it has been shown (Arnon 1956, Thomas 1957, Holt 1951) that isolated chloroplasts and their fragments can liberate oxygen in roughly the same rate as can be obtained from the intact leaf compared on a chlorophyll weight basis. The rate of photosynthetic phosphorylation for the intact leaf has not been determined, but the rates obtained (Jagendorf 1957, Allen 1958) with isolated chloroplasts is of the same order or higher than the associated CO_2 uptake by the intact leaf compared on a chlorophyll weight basis. As isolated chloroplasts characteristically show the three groups of reactions typical for photosynthesis (oxygen evolution, photosynthetic phosphorylation and CO_2 reduction), the question arises as to why, when isolated from whole cells, the chloroplasts apparently retain their power to fix CO_2 to only 1 % of their former ability, whereas their capacity to liberate oxygen is but slightly impaired.

The observations concerning the loss of activity upon isolating cellular constituents are reflected also in the questions regarding the activity and nature of the initial carboxylation reaction in photosynthesis, namely, the reaction catalyzed by carboxydismutase. This enzyme is capable of carboxylating ribulose diphosphate to form two molecules of phosphoglyceric acid. Racker (1957) has reported that the half maximal velocity of this enzymatic reaction is at a bicarbonate concentration of $2 \times 10^{-2} M$. In contrast to this value, intact plants have half saturation CO_2 concentrations (for photosynthesis) in the range of $3.5 \times 10^{-6} M$ to $2 \times 10^{-3} M$ (Rabinowitch 1951). The data of Pon (1959) show that the carboxylating activity of the extracted carboxydismutase system is not greater than 0.5 % of the CO_2 uptake rate in intact *Chlorella*. The latter calculation assumes that the enzyme accounts for approximately 10 % of the nitrogen of the cell, and is based on the enzymatic CO_2 fixation rate obtained by extrapolation to a total CO_2 concentration of $1.3 \times 10^{-3} M$. This concentration corresponds to 0.4 % CO_2 in equilibrium

with a buffer solution at pH 7.3. This concentration gives the maximum CO_2 fixation rate for *Chlorella* (Bassham, personal communication). From these low turn-over rates for carboxydismutase, there arises the problem of its relative inactivity. Several possibilities emerge: (a) the enzyme is not pure in its extracted form, (b) it has been denatured during isolation to an extent that lowers its activity to the observed value, (c) there is some essential co-factor or other enzyme necessary for full activity and which is not provided in the *in vitro* tests, or (d) some form of "active CO_2 " is the natural substrate rather than CO_2 or bicarbonate ion.

It is thus apparent that at least for some reactions, the activity of the isolated chloroplasts appears much lower than it must be in the intact cell. The reason for such a decrease in activity could be ascribed either to an environmental change, such as pH (and such other non-specific factors as are mentioned in the preceding paragraph), or to the lack of a necessary collaborator resulting from its physical separation from other cellular constituents. This latter view would regard the many particulate bodies of the cell as being more or less specialized for carrying out reactions which are essential to the overall metabolic balance of the cell. Disruption of the cell and isolation of one component, such as the chloroplast, would eliminate the interactions between cellular components present in the intact cell.

The present investigation was undertaken in an attempt to obtain experimental evidence for such interactions between various parts of a cell. The cellular components of spinach leaves were separated into three fractions (the chloroplasts, the mitochondrial fraction, and the fresh sap), and these fractions were tested, both singly and in combination, for uptake and distribution of radiocarbon and also for assimilation of various carbon-14 labelled substrates. This experiment may be viewed as an attempt to reconstruct the biochemical environment of the inside of a cell after various components of that cell have been separated and separately studied.

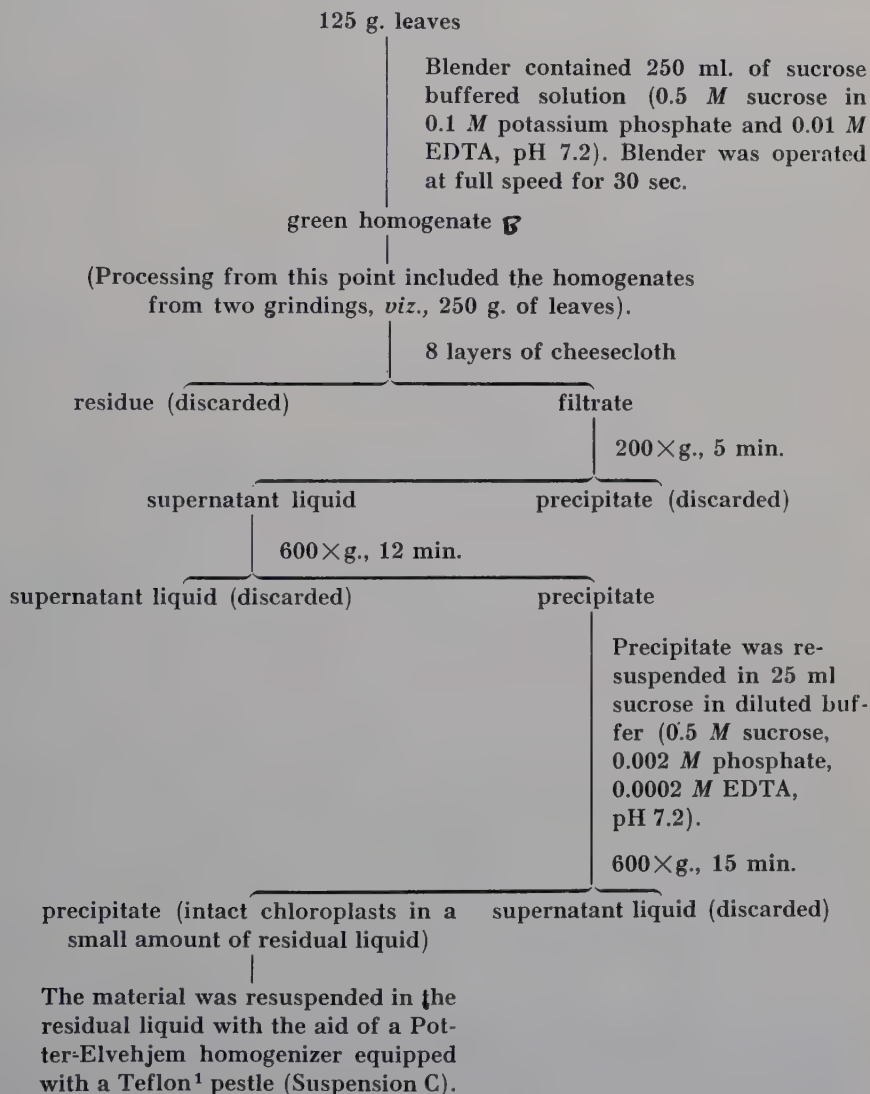
Experimental Procedure

The following abbreviations are used in this paper: EDTA, ethylenediamine tetraacetic acid; ADP, adenosine diphosphate; TPN, triphosphopyridine nucleotide; DPN, diphosphopyridine nucleotide; TPP, thiamine pyrophosphate; RuDP, ribulose-1,5-diphosphate; PGA, phosphoglyceric acid; FMN, flavin mononucleotide; UDPG, uridine diphosphoglucose; PEP, phosphoenolpyruvate.

Spinach leaves (*Spinacea oleracea*), obtained as fresh as possible from a commercial source, were used to prepare all fractions. For the preparation of whole chloroplasts, mitochondria and sap, the petioles were removed and the leaves were weighed. The leaves were washed thoroughly in cold tap water and drained as dry as possible. For the chloroplast preparation, the leaves were drained dry by "tumbling" in a

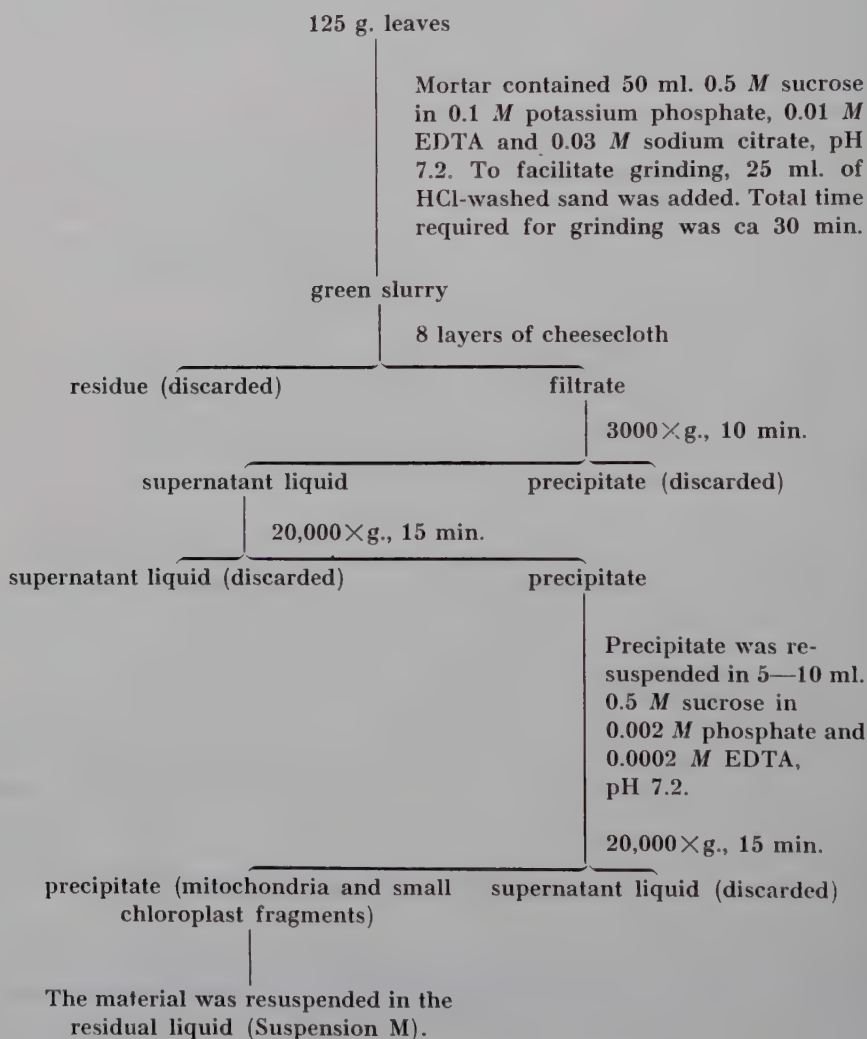
wire basket, while for the preparation of sap and mitochondria the leaves were further dried by blotting with paper towels. All subsequent operations were carried out at, or near, 0°C. All glassware and centrifugation apparatus were precooled to about 0°C unless otherwise noted.

Preparation of intact chloroplasts: Two hundred and fifty grams of leaves were used for each experiment. Grinding was performed in two batches, the leaves being cut into strips 0.5 cm. wide. All centrifugations were performed in an International Portable Refrigerated Centrifuge, Model PR-2. The flow diagram for a typical preparation is given below:



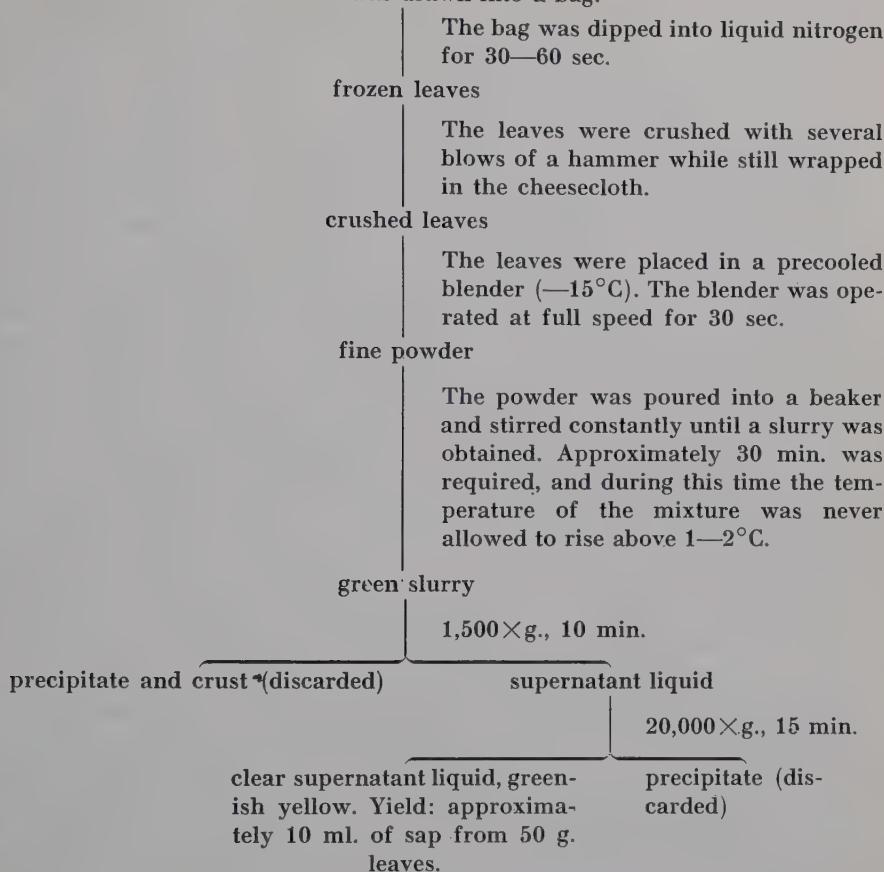
¹ Teflon is duPont's trademark for its polyfluorohydrocarbon, tetrafluoroethylene.

Preparation of the mitochondrial fraction: The leaves were cut into small pieces (ca 0.5 cm²) prior to homogenization. Low speed and high speed centrifugations were performed, respectively, in an International Portable Refrigerated centrifuge, Model PR-2 and a Spinco Ultracentrifuge, Model L. The procedure is shown in the following flow diagram:



Preparation of sap: Centrifugations were performed at low and high speeds, respectively, in an International Portable Refrigerated Centrifuge, Model PR-2, and a Spinco Ultracentrifuge, Model L, equipped with a No. 40 rotor. The leaves were cut into small pieces (ca 2 cm.²) prior to the following treatment:

150—350 g. leaves were placed in the center of five layers of cheesecloth and the cheesecloth was drawn into a bag.



Further treatment of the clear supernatant liquid (sap): A suitable aliquot portion was set aside for the preparation of boiled sap (see below). Another 5 ml. portion was adjusted to pH 7.2 at 25°C with 1 N KOH (taking careful note of the volume required). The rest of the supernatant liquid was maintained at 0°C and the pH was adjusted by the addition of a calculated amount of 1 N KOH using the volume determined in the previous step. (This operation was found necessary because the sap pH had a large temperature coefficient; thus, the pH of the sap if adjusted to 7.2 at 0°C would drop to 6.8 when the sap was allowed to warm to room temperature.) The above adjusted solution constituted Solution S.

Preparation of the boiled sap: The sap was heated in a boiling water bath for 5 min. The precipitate was removed by centrifugation and the clear, yellow supernatant liquid was allowed to cool to 25°C before the pH was adjusted to 7.2 (solution S(b)).

Photosynthesis and dark fixation experiments: The apparatus used for the photosynthesis studies was described by Moses and Calvin (in press). The temperature at

which the experiments were carried out was about 18°C; the light intensity was 2,000 f.c. The vessels employed for the dark reaction studies were 10 ml. Erlenmeyer flasks. Each flask was taped thoroughly with opaque, black adhesive tape and fitted with a rubber stopper. To insure further that no light leaked into the container through the lip of the flask, an aluminum cap was placed over the stopper and the lip. The temperature of the dark fixation vessels was about 25°C.

Loading the reaction vessels: Appropriate volumes of the four mixtures (C, S, M and S(b)) were added, singly and in combination, to each reaction vessel. The total volume in each case amounted to 1.0 ml. The exact volume of each solution or suspension is listed in the RESULTS section under the various tables. Furthermore, except where otherwise noted, 0.05 ml. of a cofactor solution was added to each flask. This solution contained, in a 0.05 ml. aliquot portion, the following materials (values are in μ moles): MgSO_4 , 4; MnCl_2 , 1.6; ADP, 1; TPN, 0.02; DPN, 0.2; TPP, 1.4; and ascorbic acid, 2.5. Some reaction vessels contained other substrates. These additions were generally limited to 0.1 ml. or less and are described in the next section. The reaction was started by the addition of 0.1 ml. of $\text{NaHC}^{14}\text{O}_3$ (0.0438 M, 1 mc/ml.), except in those cases where other radioactive substrates were added. The pH of all solutions (cofactors and substrates) was adjusted to 7.2 just prior to use. Incubation was generally carried out for 30 min. The reaction was stopped by the addition to each flask of 4 ml. of absolute alcohol at room temperature; the final concentration of alcohol was thus 80 %.

Chromatography and identification of products: The 80 % ethanolic suspension was centrifuged and the precipitate was extracted once at 50°C with 3–4 ml. of 20 % ethanol. The extracts were combined and a suitable aliquot portion (200–500 μ l) was subjected to chromatography. Chromatography was carried out on oxalic acid washed Whatman No. 4 paper with phenol-water in the first dimension and butanol-propionic acid-water in the second dimension (Benson 1955).

The radioactive areas on the chromatograms were located by exposing the paper to duPont X-ray film type 507E. Counting of C^{14} on the paper was accomplished with a Geiger-Müller tube fitted with a "Mylar" window, and flushed continuously with Q-gas (99.05 % He and 0.95 % isobutane) (Hayes 1955). For plate counting, a Nuclear-Chicago automatic counter was employed. Duplicate planchets were prepared, drying the sample together with a drop of 6 N acetic acid. The samples were spread on the aluminum plates using a rotating table and dried with a blower (Calvin 1949). Preliminary identification of labelled compounds on the paper was established by their R_f values. Confirmation was achieved by elution of the compound and rechromatography with authentic marker substances. If the compound was in the organic phosphate area of the chromatogram, it was also subjected to dephosphorylation with acid prostatic phosphatase (Schmidt 1955) and rechromatographed with authentic markers. Exact coincidence between the labelled substance and the marker was taken as the criterion of identity.

Phosphate esters were detected by spraying the paper with the reagent described by Hanes and Isherwood (1949), drying in an oven with a forced air draft at about 60°C and then exposing to sunlight. Amino acids were sprayed with 0.1 % (w/v) ninhydrin in 95 % (w/v) ethanol and the chromatograms were heated at 100°C for 5 min. Organic acids were sprayed with an 0.04 % (w/v) ethanolic solution of bromocresol green, made just alkaline with a little NH_3 . Sugars and glyceric acid were detected by dipping the chromatograms into a solution of AgNO_3 in acetone, allowing them to dry, and then spraying them with ethanolic NaOH (Trevelyan 1950). After

development of the spots, the excess AgNO_3 was washed off with a dilute ammonia solution and the papers were dried. Nucleotides and nucleosides were located by UV absorption on the paper. Those chromatograms of extracts of experiments involving sap generally had enough amino acids and reducing compounds to constitute useful markers for the spray tests.

Chlorophyll determination: Chlorophyll determinations were carried out on the various fractions which contained green material. The method consisted of extracting the material with 80 % acetone, clarifying the acetonic solution by centrifugation and reading the supernatant liquid in a spectrophotometer in the wavelength range from 640 m μ to 700 m μ . The details of this procedure are described by Arnon (1949).

For the determination of chlorophyll in the intact leaves, it was found that not all of the green material was extracted by acetone. In this case ethanol was substituted and heat (steam bath) was applied to hasten the extraction. The concentrated alcoholic solution obtained in this manner was diluted in 80 % acetone and treated as indicated above.

Protein determination: The protein content of a sample was determined colorimetrically on the basis of a combination of colors from the biuret reaction and the tyrosine-Folin color reaction. The method is described by Lowry (1951). The reference protein used to set up the standard curve was obtained by dilute phosphate buffer extraction of the chloroplasts. This extract was subjected to exhaustive dialysis followed by protein determination on a weight basis.

Results

While both the chloroplast and the sap preparations were able to fix carbon dioxide alone, the most striking effect noticed in these experiments on the activity of the chloroplasts was caused by the addition of the cell sap fraction. This is illustrated by the data shown in Table 1, from which it can be seen that chloroplasts suspended in sucrose fixed 7.3×10^6 dpm (disintegrations per minute), sap fixed 1.5×10^6 dpm, and the combination of chloroplasts and sap fixed 27×10^6 dpm. The addition of the sap thus brought about a fixation of radioactive carbon that was over three times that to be expected from a simple addition of the fixation of each component separately. The distribution of the fixed carbon into the alcohol-soluble compounds is also shown in Table 1, and, in addition, representative radioautograms are shown in Figure 1.

The distribution of the radiocarbon fixed by the chloroplasts suspended in sucrose was very different from that fixed by the sap fraction alone. The former showed over 70 % of the fixed soluble radioactivity in the sugar phosphate and only 15 % in the amino acids, while the sap fixed close to 50 % of the carbon-14 in the amino acids only 33 % in the sugar phosphates. The percentage of activity in the sugar phosphates from the sap fixation (33 %) was higher than is usually observed in dark fixation experiments by photo-

synthetic organisms or in CO_2 fixation by nonphotosynthetic organisms (Moses, in press). This may indicate either that labelled aspartic acid was being converted to PGA, giving rise to labelled PGA by a nonphotosynthetic route, or that some of the chlorophyll which was still found in this fraction may have mediated the fixation of some carbon dioxide via a photosynthetic mechanism. The latter possibility is unlikely in view of the results obtained by the addition of the mitochondrial fraction to the sap, as the amount of chlorophyll present in the mitochondrial fraction was greater than that in the sap alone. Neither the total uptake of CO_2 nor the distribution of the radioactivity shown by the sap fraction was altered by the addition of the mitochondrial fraction.

The increase in fixation upon adding the sap fraction to chloroplasts was not caused by an inorganic constituent, as boiling the sap for 5 min. destroyed its ability to increase the total fixation of labelled carbon dioxide when added to the chloroplast suspension (Table 1). The most noticeable difference between the effects of fresh and boiled sap on the distribution of C^{14} fixed by the chloroplasts was in the relative amounts found in the sugar phosphates and amino acids. With chloroplasts and fresh sap some 71 % of the total soluble radioactivity was present in the phosphates, whereas chloro-

Key to Table 1:

C: Suspension C, 0.1 ml. (0.61 mg. chlorophyll and 8.9 mg. protein) plus 0.9 ml. 0.5 *M* sucrose buffered at pH 7.2.

S: Solution S, 1.0 ml. (0.03 mg. chlorophyll and 27.4 mg. protein).

M: Suspension M, 0.1 ml. (0.44 mg. chlorophyll and 5.2 mg. protein) plus 0.9 ml. 0.5 *M* sucrose buffered at pH 7.2.

C+S: Suspension C, 0.1 ml. plus 0.9 ml. Solution S. (This corresponds to between 1.2 and 5.0 times the ratio of sap to chlorophyll which may be in the leaves. The uncertainty lies in the amount of sap in the leaf.)

C+M+S: Suspension C, 0.1 ml. plus 0.1 ml. suspension M plus 0.8 ml. Solution S.

C+S(b): Suspension C, 0.1 ml. plus 0.9 ml. Solution S(b) (9.9 mg. protein).

All other details are described in the Experimental Procedure section.

Disintegrations per minute: This represents the total C^{14} fixed in the alcohol-soluble portion.
cpm: Counts per minute on the paper chromatogram calculated for the entire sample (uncorrected for geometry, self-absorption and coincidence).

%: Percent of the total soluble activity calculated by the summation of all the radioactive spots on the chromatogram.

Phosphates: Sum of the listed phosphates, including those not listed (phosphoglycolic acid and triose phosphate).

Amino acids: Sum of the listed amino acids, including those not listed (serine, citrulline, valine, glutamine and other unidentified ninhydrin-positive spots).

Free sugars: Sum of the listed sugars only.

Organic acids: Sum of the listed acids including citric acid, pyruvic acid, glycolic acid, succinic acid and fumaric acid.

P: Present, but not separated from glycine and therefore counted together with glycine.

Table 1. Total uptake and distribution of carbon-14 from labelled carbon dioxide by various combinations of cellular constituents of spinach.

Reaction mixture	C		S		M		C + S		C + M + S		C + S (b)		C + M + S	
	30 min. light	7.3 $\times 10^6$	30 min. light	1.5 $\times 10^6$	30 min. light	0.05 $\times 10^6$	30 min. light	27 $\times 10^6$	30 min. light	16 $\times 10^6$	30 min. light	7.7 $\times 10^6$	30 min. light	2.5 $\times 10^6$
Disintegrations per minute	cpm $\times 10^{-3}$	%	cpm $\times 10^{-3}$	%	cpm $\times 10^{-3}$	%	cpm $\times 10^{-3}$	%	cpm $\times 10^{-3}$	%	cpm $\times 10^{-3}$	%	cpm $\times 10^{-3}$	%
Compound:														
Diphosphates	17	3.4	—	—	—	—	140	8.4	15	1.8	48	12	0.8	—
UDPG	23	4.9	0.4	0.5	—	—	20	1.2	6.1	0.7	—	—	—	0.3
Monophosphates	210	46	2.8	3.1	—	—	420	25	180	22	160	39	44	31
PGA	78	17	21	23	59	1.0	490	29	270	33	170	42	36	25
PEP	—	—	5.2	5.8	—	—	100	5.9	62	7.5	2	0.5	7.7	5.5
Σ Phosphates	330	72	29	33	59	1.0	1,200	69	550	67	380	93	89	62
Aspartic acid	21	4.5	43	48	42	0.7	210	12	110	14	5.2	1.3	6.8	4.8
Alanine	8.6	1.8	1.1	1.2	—	—	69	4.1	36	4.3	5.8	1.4	12	8.3
Glutamic acid	36	7.8	—	—	—	—	—	—	—	—	—	—	—	0.2
Glycine	66	14	44	49	42	0.7	310	18	180	22	7.4	1.8	11	8.2
Σ Amino acids	4.0	0.9	—	—	—	—	21	1.2	—	—	—	5.2	33	23
Fructose	P	P	—	—	—	—	27	1.5	P	P	P	P	2.4	1.6
Glucose	—	—	—	—	—	—	6.8	0.4	—	—	—	—	—	—
Sucrose	11	2.3	—	—	—	—	11	0.6	—	—	—	—	2.6	1.8
Maltose	15	3.2	—	—	—	—	58	3.8	—	—	—	—	4.9	3.4
Σ Free sugars	—	—	2.1	2.3	—	—	59	3.5	30	3.6	—	—	11	8.2
Glycerin acid	19	4.0	13	15	—	—	74	4.4	54	6.6	4.9	1.2	4.5	3.4
Malic acid	52	11	16	18	—	—	160	9.3	90	11	9.0	2.2	16	3.2
Σ Organic acids	—	—	—	—	—	—	—	—	—	—	—	—	—	11

plasts and boiled sap fixed 93 % of the soluble radioactivity into these compounds, with PGA alone accounting for 42 %.

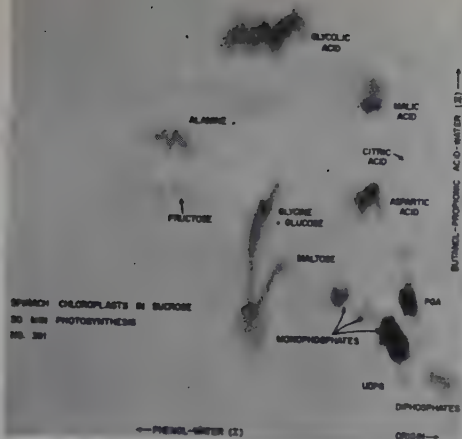
The mitochondrial fraction showed very little fixation of CO_2 (0.4 % that of the chloroplast fraction) with the fixed radioactivity being restricted mainly to PGA and aspartic acid (Table 1). This fraction did not alter the distribution of radioactivity when added to chloroplasts or sap, but did consistently depress the total amount of radioactive carbon dioxide fixed by the chloroplast-sap mixture. The reason for the decrease in the rate of carbon dioxide fixed by the chloroplast-sap mixture following the addition of mitochondria is not clear. The effect may represent a physical phenomena of surface adsorption of some required enzyme or reactant on the mitochondria.

The omission of the cofactor supplement resulted in a marked decrease in the total fixation of CO_2 by the chloroplast-sap mixture. The amount of radiocarbon fixed was only 14 % of that fixed by the chloroplast and sap mixture when the cofactor supplement had been added (Table 1). The effects of the individual components of this cofactor supplement were not separately studied, and it is therefore not possible to state which of the substances in the cofactor mixture was responsible for the large stimulation of fixation.

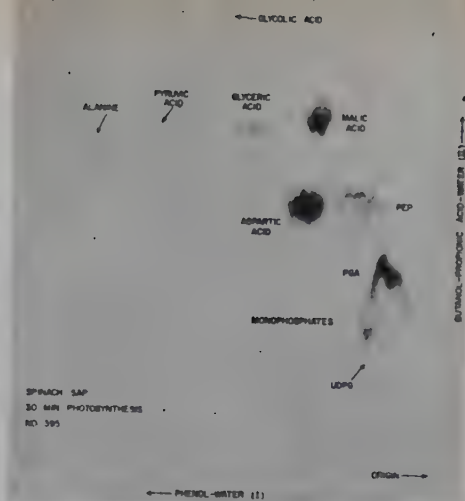
That the fixation of CO_2 in these experiments does actually represent a photosynthetic uptake of CO_2 and not merely dark reactions is shown by the low total fixation of C^{14} by the combination of chloroplasts, sap and mitochondria when incubated with labelled CO_2 in the dark for 30 minutes (Table 1). Over 86 % of the fixed soluble radioactivity was incorporated into aspartic acid and malic acid. These two compounds characteristically incorporate radioactive carbon dioxide via well known dark reaction mechanisms (Vishniac 1957, Meister 1957). In another experiment, using chloroplast-sap mixture the fixation of carbon dioxide in the light was some 25 times greater than that in the dark.

In order to obtain more information on the effects of sap on the light fixation of carbon dioxide by chloroplasts, the following two experiments were performed: (i) The fixation of CO_2 by the chloroplast and sap mixture was measured at 1, 3, 10 and 30 min. after the introduction of the radioactive bicarbonate, and (ii) the effect of varying the amount of sap was determined for fixation times of 30 min. The results from the kinetic experiment are shown in Table 2 and Figures 2a and 2b together with radioautograms in Figure 3. The fixation of CO_2 by this system was approximately linear with time, and PGA was the dominant radioactive material, especially for the shorter exposure periods to radioactive carbon dioxide. A plot of the radioactivity with time in each compound as a percentage of the total soluble radioactivity shows that PGA and the diphosphate area both had negative slopes, but of these the PGA contained the most activity, having 61 % of the

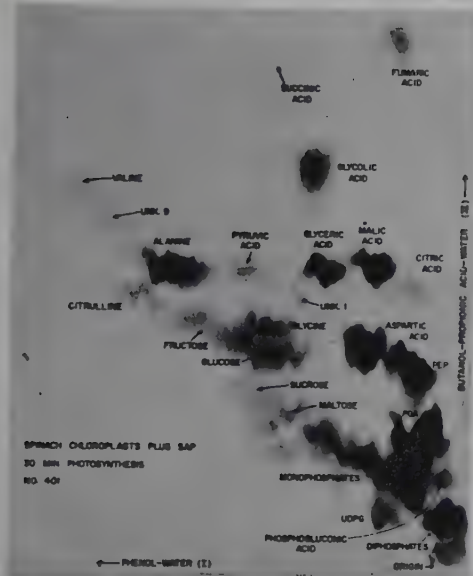
a



b



c



d

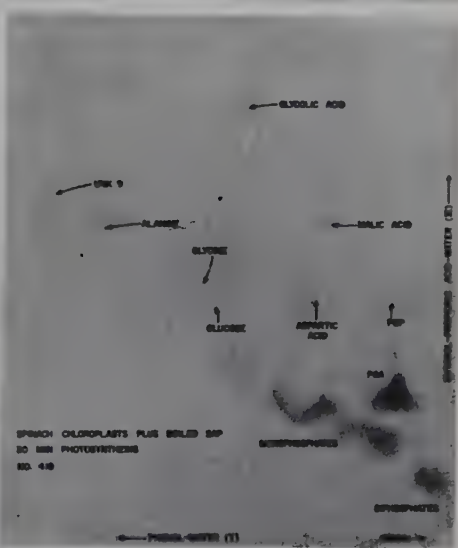


Figure 1. Radioautograms showing distribution of radioactive carbon by various cellular constituents of spinach. (a) chloroplasts in sucrose; (b) sap; (c) chloroplasts plus sap; (d) chloroplasts plus boiled sap; (e) chloroplasts plus mitochondrial fraction plus sap in the dark for 30 min. For experimental details see Table 1.

Table 2. Total uptake and distribution of carbon-14 by chloroplast-sap mixture after varying times of exposure to radioactive bicarbonate.

Exposure time	1 min.		3 min.		10 min.		30 min.	
	0.37×10^6		1.5×10^6		6.9×10^6		29×10^6	
Disintegrations per minute	cpm $\times 10^{-3}$	%	cpm $\times 10^{-3}$	%	cpm $\times 10^{-3}$	%	cpm $\times 10^{-3}$	%
Compound:								
Diphosphates.....	2.3	13	4.4	6.2	12	3.9	43	3.1
UDPG	—	—	—	—	2.4	0.8	17	1.2
Monophosphates ...	2.0	11	10	14	38	13	280	21
PGA.....	10	61	41	58	170	57	500	36
PEP.....	0.3	1.8	2.4	3.4	20	6.7	110	7.9
Σ Phosphates	15	88	58	81	240	81	950	69
Aspartic acid.....	1.4	7.9	5.2	7.3	18	6.1	180	13
Alanine	—	—	1.1	1.5	3.4	1.1	48	3.5
Glycine	—	—	1.0	1.4	4.2	1.4	45	3.3
Σ Amino acids	1.4	7.9	8.6	12	38	13	310	22
Sucrose	—	—	—	—	—	—	10	0.8
Σ Free sugars	—	—	—	—	—	—	10	0.8
Glyceric acid.....	—	—	1.5	2.1	5.4	1.8	44	3.2
Malic acid	0.7	4.2	3.0	4.2	13	4.2	42	3.0
Σ Organic acids ...	0.7	4.2	4.8	6.7	21	6.9	100	7.6

Key to Table 2:

Chloroplast-sap mixture: Suspension C, 0.1 ml. (0.68 mg. chlorophyll and 9.2 mg. protein) plus 0.9 ml. solution S (0.03 mg. chlorophyll and 17 mg. protein). The ratio of sap to chlorophyll lies between 4.3 and 1.0 that of the ratio which may be present in leaves.

Disintegrations per minute: Total C^{14} fixed including both the alcohol soluble and insoluble fractions.

Explanations for the rest of the descriptions are given under Table 1.

soluble activity after one minute of fixation time (Figure 2c). When the amount of sap which was added to the chloroplast suspension was varied, the total fixation was directly proportional to the amount of sap added (Figure 2b).

Except in the 30 minute dark fixation (Table 1), no labelled glutamic acid was found in these experiments. It was therefore of interest to see if radioactive carbon would be incorporated into glutamic acid in the dark period following a period of photosynthesis with radioactive CO_2 , as has been reported for *Chlorella* (Bassham 1956). The results (Table 3) show that no radioactive glutamic acid was discernible in the chloroplast and sap-mixture after 5 min. photosynthesis with radioactive CO_2 , followed by a 5 min. period of darkness.

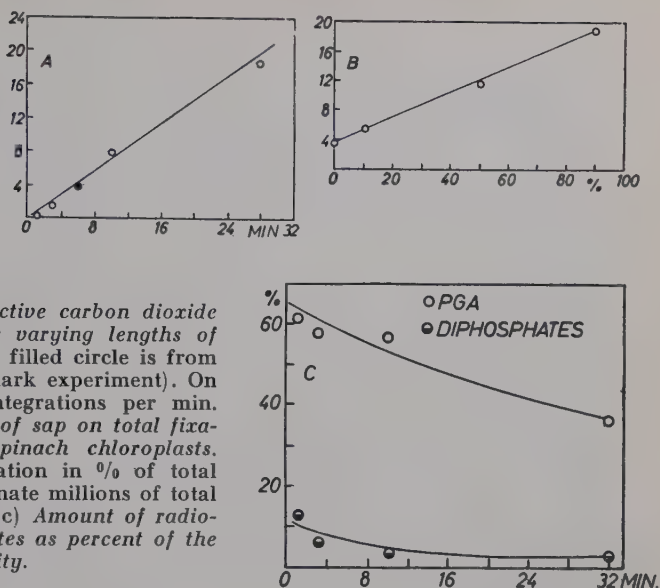


Figure 2 (a) Uptake of radioactive carbon dioxide by chloroplasts plus sap after varying lengths of time with labelled carbon (The filled circle is from the 5 min. light, then 5 min. dark experiment). On the ordinate millions of disintegrations per min. (b) Effect of varying amounts of sap on total fixation of carbon dioxide by spinach chloroplasts. On the abscissa sap concentration in % of total incubation volume. On the ordinate millions of total disintegrations fixed per min. (c) Amount of radioactivity in PGA and diphosphates as percent of the total alcohol soluble radioactivity.

The chloroplast-sap mixture did metabolize labelled RuDP. However, since the labelled RuDP was itself contaminated with a variety of other labelled compounds normally found in algae it was impossible to make a qualitative estimation of the fate of the labelled RuDP. Attempts were made to determine whether RuDP was metabolized by the sap alone or the chloroplasts alone (Table 4). Addition of unlabelled RuDP to a chloroplast suspension in the dark resulted in an increase fixation of labelled carbon dioxide of about 800,000 dpm over that of the dark fixation of a chloroplast suspension in the absence of RuDP. The most pronounced effect on the distribution of fixed carbon-14 following the addition of unlabelled RuDP was that most of the increase in C^{14} fixed was accounted for by an increase in labelled PGA.

Neither the sap fraction nor the chloroplasts were able to metabolize labelled PGA very rapidly (Table 3). The sap fraction did show incorporation of carbon-14 into PEP, glyceric acid, alanine and citric acid. Metabolism of labelled PGA by the chloroplasts was slow, and small amounts of radioactivity only, were found in the hexose monophosphates, maltose, and glycolic acid.

Studies with citric-1,5- C^{14} as a substrate for Krebs cycle activity showed the formation of glutamic, α -ketoglutaric, and other acids by sap, but not by chloroplasts.

In several experiments with the chloroplasts and sap mixture at pH 6.1, there was a marked decrease in the total fixation of CO_2 to about 1 % of

Table 3. *Uptake and distribution of radioactive carbon by sap and chloroplasts under various conditions.*

Reaction mixture ...	C + S		C + PGA-C ¹⁴		S + PGA-C ¹⁴	
Conditions	5 min. light, then 5 min. dark		30 min. light		30 min. light	
Disintegrations per minute	4.0×10^6					
	cpm $\times 10^{-3}$	%	cpm $\times 10^{-3}$	%	cpm $\times 10^{-3}$	%
Compound:						
Diphosphates.....	0.8	0.4	—	—	—	—
UDPG	—	—	—	—	—	—
Monophosphates ...	15	8.3	20	13	—	—
PGA.....	89	50	130	83	190	76
PEP.....	14	8.1	—	—	28	11
Σ Phosphates	120	67	150	98	220	87
Aspartic acid	22	12	—	—	—	—
Alanine	9.6	5.0	—	—	9.1	3.6
Glycine	3.3	1.9	—	—	—	—
Σ Amino acids	42	23	—	—	9.1	3.6
Maltose	—	—	2.6	1.6	—	—
Σ Free sugars	—	—	2.6	1.6	—	—
Glyceric acid.....	3.6	2.0	—	—	24	9.6
Malic acid	11	6.4	—	—	—	—
Σ Organic acids ...	17	9.8	1.0	0.7	25	10

Key to Table 3:

C+S: This chloroplast-sap mixture is identical to that in Table 2.

C+PGA-C¹⁴: Suspension C, 0.1 ml. (0.68 mg. chlorophyll and 9.2 mg. protein) plus carboxyl labelled PGA prepared by reacting RuDP with NaHC¹⁴O₃ in the presence of carboxy-dismutase (Mayaudon, 1957). The concentration of the PGA used in this experiment was unknown. Unlabelled bicarbonate was added in place of radioactive bicarbonate.

S+PGA-C¹⁴: Solution S, 1.0 ml. (0.03 mg. chlorophyll and 19 mg. protein) plus PGA-C¹⁴. Disintegrations per minute: Total C¹⁴ fixed in the alcohol soluble fraction only.

Explanations for the rest of the descriptions are given in Table 1. Other details of the experiment are described in Experimental Procedures section.

that obtained at the pH 7.2. The pattern of carbon-14 distribution at pH 6.1 was also very different than that found at pH 7.2 (Figure 4). In this experiment the alcohol-soluble compounds were separated by use of exchange resins into a neutral fraction and an ionized fraction. The neutral fraction, consisting largely of glucose, fructose, sucrose, maltose, and various polysaccharides of glucose, comprised about 50 % of the total fixed radioactivity. The ionized fraction, which consisted mostly of the phosphorylated compounds, amino acids and organic acids, contained the remaining 50 % of the fixed activity. When the experiment was performed at pH 7.2, the free

Table 4. *The effect of added RuDP on the fixation of C¹⁴O₂ by chloroplasts in the dark, 30 min.*

Reaction mixture	C		C + RuDP	
Disintegrations per minute	0.43×10^6		1.2×10^6	
	cpm $\times 10^{-3}$	%	cpm $\times 10^{-3}$	%
Compound:				
Diphosphates.....	—	—	—	—
UDPG.....	—	—	—	—
Monophosphates.....	—	—	—	—
PGA.....	19	55	47	81
PEP.....	—	—	—	—
Σ Phosphates.....	20	58	49	85
Aspartic acid.....	6.0	17	3.5	6.1
Alanine.....	—	—	1.8	3.0
Glycine.....	—	—	0.2	0.4
Σ Amino acids.....	10	30	5.5	9.5
Maltose.....	—	—	—	—
Σ Free sugars.....	—	—	—	—
Glyceric acid.....	—	—	—	—
Malic acid.....	2.8	8.0	3.4	5.9
Σ Organic acids.....	4.0	12	3.4	5.9

Key to Table 4:

C: Suspension C, 0.1 ml. (0.68 mg. chlorophyll and 9.2 mg. protein) plus 0.9 ml. 0.5 M sucrose buffered at pH 7.2.

C+RuDP: Same as above except that 0.01 ml. of RuDP solution containing about 0.1 μ mole of RuDP was added. The RuDP was prepared according to the method described by Horecker *et al.* (1956).

Disintegrations per minute: Total C¹⁴ fixed in the alcohol soluble fraction only.

sugars (corresponding to the neutral fraction in the above experiment contained only 2—3 % of the total fixed radioactivity).

Another striking effect caused by lowering the pH of the chloroplast suspension from 7.2 to 5.0 concerned the ability of these particles to carry out transamination. This effect was tested by incubating the sap or chloroplasts fractions with pyruvic acid-3-C¹⁴ and unlabelled alanine. At pH 7.2, the chloroplasts showed strong transaminase activity as witnessed by the large amount of radioactivity incorporated into the alanine, whereas at pH 5.0 no radioactivity was evident in alanine. The sap exhibited strong transamination at pH values of 7.2 and 6.1.

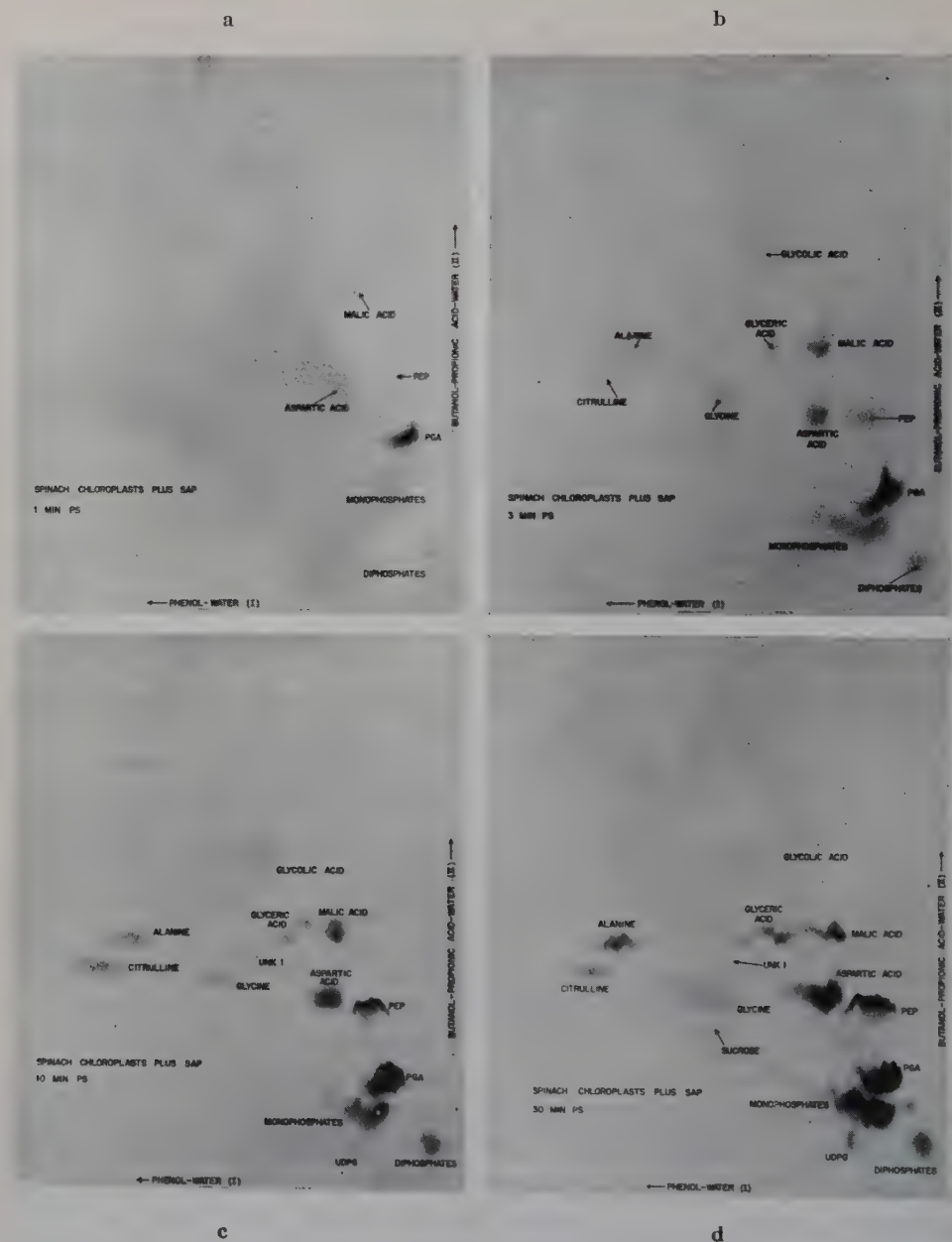


Figure 3. Radioautograms showing pattern of carbon-14 distribution by chloroplast-sap mixture after varying lengths of time of exposure to labelled carbon dioxide (see Table 2) (a) 1 min.; (b) 3 min.; (c) 10 min.; (d) 30 min.

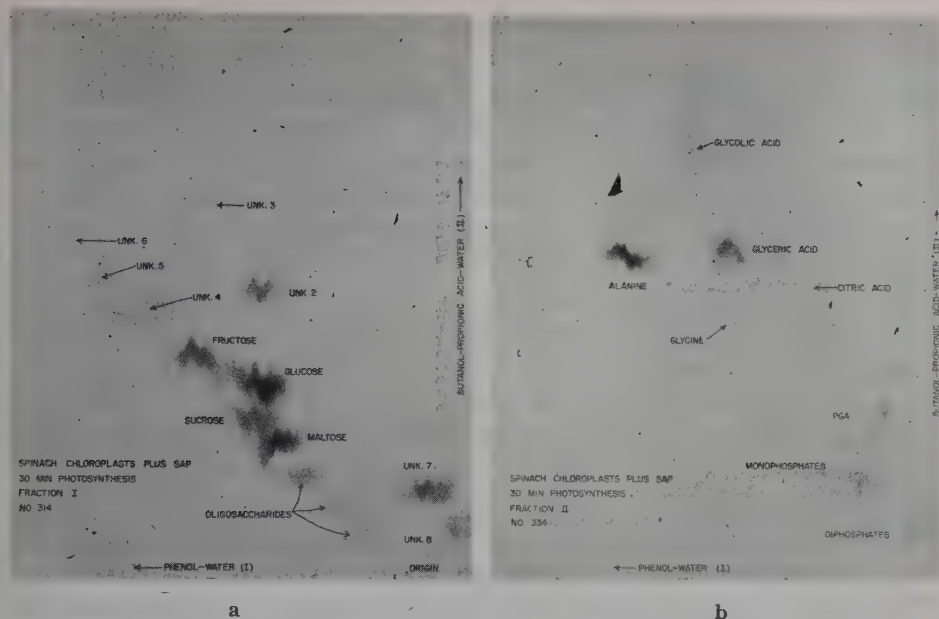


Figure 4. Radioautograms showing pattern of distribution of radioactive carbon by chloroplast plus sap mixture at pH 6.1. (a) The neutral fraction (Fraction I); (b) the ionized fraction (Fraction II).

Discussion

Although both the chloroplast and sap fractions were very active in assimilating carbon dioxide, especially when mixed together, the mitochondrial fraction had little effect both on the rate of carbon dioxide fixation (a slight depressant effect was noted) and on the distribution of the fixed carbon. This mitochondrial fraction, which was defined as that particulate material which centrifuged down between $3000\times g$. and $20,000\times g$., most certainly was not a pure preparation of mitochondria, but contained a mixture of particulate matter. This fraction was tested manometrically for oxygen uptake upon addition of a Krebs cycle intermediate (citric acid), and it was found to be active to some degree but not to the extent which would be expected from reported data on mitochondrial activity (Ohmura 1955). The activity of this fraction was also tested by the addition of labelled citric acid. Its ability to carry on transamination was also tested by addition of labelled pyruvic acid and unlabelled alanine. In both cases there was little or no conversion of the added substrates by the mitochondria.

It is thus apparent that this fraction must have lost activity during the

isolation procedure, and the largely negative results obtained upon addition of it to samples of chloroplasts and sap must be interpreted with caution. The tendency of mitochondria to depress the total fixation of CO_2 may represent a physical adsorption of some factor or enzyme by the particulate matter in the mitochondrial fraction. In studies such as those reported in the present communication, it would be of great value to be able to obtain mitochondria uncontaminated by green chloroplast fragments, and to be able to demonstrate manometrically that the particles possessed strong citric acid cycle activity. Until this is done, it is hard to extrapolate from these experiments to the possible interaction of these particles with the chloroplasts in the intact cell.

The most important observation that emerges from the data is the strong stimulating effect on the total fixation of CO_2 obtained by adding fresh sap to the isolated chloroplasts. This effect is not explicable, based on the knowledge available to us today, in terms of deficiencies of essential cofactors or in terms of osmotic or pH effects, but rather by some catalytic interaction of unknown nature between the two preparations.

The cofactor supplement which was added contained the factors which are commonly accepted as showing a stimulation of chloroplast activity, with the possible exception of a flavin derivative and Vitamin K. It is unlikely that either a flavin or Vitamin K was responsible for the observed effect because they would be expected to resist heating to 100°C for 5 minutes. In fact it was shown that boiling the sap destroyed its ability to stimulate chloroplast activity. From the knowledge that the rate of carbon dioxide fixation by a chloroplast-sap mixture remained approximately linear for 30 min., and that the stimulation of chloroplast activity by sap was directly proportional to the amount of sap added, it appears unlikely that the sap was merely providing some metabolite or reactant which is necessary for chloroplast activity unless this reactant were of a catalytic nature. It is possible to conceive of the sap providing a heat-labile cofactor or enzyme which can partially overcome a bottleneck limiting the rate of entry of CO_2 into the photosynthetic cycle.

This could be visualized in one of the following three ways. First, the sap may be adding a substance which serves as an activator of carbon dioxide, the activated carbon dioxide being the natural substrate for the carboxylation of ribulose diphosphate rather than bicarbonate ion or dissolved carbon dioxide. It is known that the turnover rate of carboxydismutase, when measured after extraction from the cells, is too low to account for the rate at which it must be turning over in the intact cell if the photosynthetically-fixed carbon dioxide is indeed being mediated by this enzyme (see Table 5). One of the hypotheses that has been suggested to explain this is that in the *in vitro* experiments with the enzyme, free CO_2 or bicarbonate has been added as the

Table 5. Comparison of carbon dioxide fixation rates *in vivo* and *in vitro* systems.

Plant or preparation	Investigator	Rate of CO ₂ fixation at 1.3×10^{-3} M, total CO ₂ species. ¹ μmole/min. · mg. protein	% of <i>in vivo</i> CO ₂ fixation rate
Intact <i>Chlorella pyrenoidosa</i>	Bassham (personal communication)	0.4 ²	100
Enzyme preparation from <i>Spinacea oleracea</i>	Weissbach (1956)	0.27 ³	68
Enzyme preparation from <i>Spinacea oleracea</i>	Racker (1957)	0.044 ³	11
Enzyme preparation from <i>Spinacea oleracea</i>	Jakoby, <i>et al.</i> (1956)	0.019 ⁴	4.8
Enzyme preparation from <i>Tetragonia expansa</i>	Pon (1959)	0.02 ⁴	5.0

¹ This concentration is equivalent to 0.4 % in equilibrium with a pH 7.3 buffer.

² The CO₂ fixation rate for the *in vivo* system assumes that 20 % of the wet weight of algae is equal to the dry weight, 50 % of the latter being equal to the weight of the protein in the cells. It should be emphasized that these comparisons also assume that all of the protein within the cell constitutes the carboxylation enzyme.

³ These fixation rates were calculated by substituting the values for the Michaelis constant for bicarbonate and the maximum velocity of the reaction (quoted in the respective references) into the Michaelis-Menten equation.

⁴ These values were obtained by assuming that the rate of fixation is directly proportional to the bicarbonate concentration.

substrate, and in the intact cell there may be some 'active CO₂' complex which serves as the substrate for the carboxylation of ribulose diphosphate. (Flavin, Castro-Mendoza and Ochoa 1956, Bachhawat, Woessner and Coon 1956, Metzner, Metzner and Calvin 1958).

The second way in which the sap could be stimulating the fixation of CO₂ by the chloroplasts would be if it provided some factor which increased photosynthetic phosphorylation. In the photosynthetic cycle there are two reactions which require high-energy phosphate: the reduction of PGA and the phosphorylation of ribulose monophosphate to RuDP. The present data do not permit a firm decision between these two hypotheses, but the distribution of radioactivity would speak more strongly for the latter alternative. When the diphosphate area was treated with acid phosphatase, most of the radioactivity was found in the fructose and glucose, with about 10–15 % in ribulose. If the carbon dioxide substrate were limiting the rate of entry of CO₂ into the cycle, it would be expected that RuDP would accumulate and be the major constituent of the diphosphate area. This is actually found in *Chlorella* or *Scenedesmus* under low CO₂ pressure (Wilson and Calvin 1955).

Table 6. Carbon dioxide fixation by various chlorophyll-containing materials.

Materials	Investigator	Max. fixation rate $\mu\text{mole CO}_2/\text{min.} \cdot \text{mg. chlorophyll}$	Final conc. total species of CO_2 , i. e. $(\text{CO}_2) + (\text{HCO}_3^-)$	Remarks
Intact chloroplasts in sap	This paper	2.9×10^{-2}	$3.8 \times 10^{-3} M$	pH 7.2
Intact chloroplasts in NaCl	Allen, <i>et al.</i> (1955)	4.2×10^{-3}	$5.0 \times 10^{-4} M$	pH 7.2
Intact <i>Chlorella pyrenoidosa</i>	Hill and Whittingham (1955)	3.1	Unknown	
Intact <i>Chlorella pyrenoidosa</i>	Bassham (personal communication)	4.0	(0.4 %) = $1.3 \times 10^{-3} M$	Assumed A. Q. = 1.0; pH 7.3
<i>Sambucus nigra</i> green leaves	Willstätter (1951)	2.5	(5 % CO_2)	
<i>Sambucus nigra</i> yellow leaves	Willstätter (1951)	44	(5 % CO_2)	
<i>Helianthus annuus</i>	Willstätter (1951)	5.3	(5 % CO_2)	
Intact <i>Spirogyra</i>	Thomas (1957)	1.5	(5 % CO_2)	A. Q. = 1.0
<i>Spirogyra</i> chloroplast fragment	Thomas (1957)	8.9	(5 % CO_2)	A. Q. = 1.0

¹ A.Q. = assimilatory quotient (the ratio of CO_2 to O_2).

If, on the other hand, the level of ATP were the limiting factor, one would expect the reduction of PGA to triose to proceed slowly and the amount of labelled RuDP to be low. Both of these circumstances were found in the chloroplast preparations. It should be recalled here that photosynthetic phosphorylation proceeds at a rapid rate in isolated chloroplasts (pH 8; see Table 7) (Allen, Whatley and Arnon 1958). It is quite possible, however, that photosynthetic phosphorylation was limiting in this case because of pH differences or the lack of some factor. With this possibility in mind, a third alternative must be considered, namely, that the rate of formation of reduced pyridine nucleotide was the limiting factor.

Using the value of 3.0 $\mu\text{moles CO}_2$ fixed/min. photosynthesis and per mg. chlorophyll for *Chlorella*, the spinach chloroplast preparations fixed CO_2 at a maximum rate about 1 % that of *Chlorella* when compared on a mg. chlorophyll basis. The actual amount of CO_2 fixed by the spinach chloroplasts was calculated from the specific activity of the bicarbonate introduced and the total uptake of radiocarbon (Table 6).

The question can then be raised as to what is limiting the ability of the chloroplasts to fix and reduce CO_2 . It has been seen that the sap, which increased the total fixation markedly, did not significantly alter the pattern of

Table 7. *Maximum initial rate of Hill reaction and rate of photolysis.*

Preparation	Investigator	Oxidant	Temp., °C	Max. initial rate of Hill reaction $\mu\text{mole}/\text{O}_2 \cdot \text{min.} \cdot \text{mg. chlorophyll}$
Intact spinach chloroplasts	Arnon, <i>et al.</i> (1956)	<i>p</i> -benzoquinone	15	0.75
Intact <i>Chlorella pyrenoidosa</i>	Hill and Whittingham (1955)	(CO ₂)		3.1
<i>Spirogyra</i> , chloroplast fragment	Thomas (1957)	(CO ₂)	18	3.1
<i>Spirogyra</i> , intact	Thomas (1957)	(CO ₂)	18	1.5
Chloroplast suspension from <i>Phytolacca americana</i>	Holt, <i>et al.</i> (1951)	2,6-dichlorobenzene indophenol		1.6

Rate of photosynthetic phosphorylation with dilute NaCl-washed chloroplast fragments (Allen, *et al.* 1958): 8.5 μmole inorg. phosphate esterified/min. \cdot mg. chlorophyll.

carbon-14 distribution. This may be interpreted to mean that whatever was limiting the ability of the chloroplasts to fix CO₂ before addition of the sap was still operative, even after addition of the sap. If the addition of the sap removed one limitation completely, the new limitation would most likely cause some shift in the incorporation patterns. It is evident that in disrupting the cells and recombining only the chloroplast and sap fractions, not only is the overall organization of the cell destroyed but there is a possibility that the essential cofactors, etc. may have been leached out of the chloroplasts during separation. To obtain more definitive answers as to the nature of the decrease in photosynthetic ability, it would be helpful to have data from experiments in which the particles were isolated in various ways. It would also be of interest to know the photosynthetic ability of a mash of spinach leaves when the cells were all disrupted but before the actual separation of any components.

Fager (1952) has actually performed experiments with macerates of spinach leaves. Unfortunately, the specific activity of the radioactive carbonate used by him is unknown and therefore it is not possible to calculate the rate of carbon dioxide fixation of the preparation. The experiments, however, brought out two significant facts: (i) The separated chloroplast material containing chloroplasts, grana, etc. did not fix any carbon dioxide, but when this chloroplast material was recombined with the cell sap, the mixture then exhibited the same CO₂ fixing ability as did the complete leaf macerate; (ii) up to 60 % of the radioactive tracer was incorporated by this macerate in the PGA fraction.

It should be pointed out that the question of transport of 'active CO_2 ' through the cytoplasm to the chloroplast remains undecided. Also unsettled is the question as to how much of the products of photosynthesis leaves the chloroplast during optimal conditions for photosynthesis, and the nature of the molecules able to enter and leave the chloroplasts. It is thus possible to conceive of the chloroplast as having limited biosynthetic capabilities, necessitating exchange of substances between the chloroplast and other biosynthetic regions of the cell.

Upon examination of the data showing the distribution of radioactive carbon by chloroplasts with and without the presence of sap, it appears that the sap affected to some degree the conversion of the intermediates involved in the photosynthetic cycle to such other metabolites as amino acids and organic acids. It is possible that this effect of the sap is accomplished by a component of the sap which can enter the chloroplast, but it is more likely that some component in the chloroplast is moving out of the chloroplast and undergoing further conversion in the sap.

It is interesting to note the presence of some labelled phosphogluconic acid in the product of photosynthesis by both chloroplasts alone as well as by the chloroplast-sap combination, (Figures 1 a and 1 c). This suggests that the pentose cycle for the oxidation of glucose is operating in these preparations and might contribute (Wood 1955) to the redistribution of the label originally present in carbon atom three into carbon atoms one and two of glucose (Gibbs and Cynkin 1958 b).

The distribution of the fixed radiocarbon by the chloroplast-sap mixture was sensitive to pH change. When the pH was changed from 7.2 to 6.1, much more radioactivity was incorporated into the free sugars and oligosaccharides. The same observation has been made by Ouellet (1952) with intact *Scenedesmus*. His results showed that the lower the pH (down to pH 1.6), the greater was the incorporation rate of radiocarbon into sucrose and polysaccharide material. Since the pH optima of the enzyme systems in plants are not known, no definite correlations can be made between the observed patterns and the pH sensitivity of specific enzymes.

These attempts to 'reconstruct' the biochemical environment of a spinach leaf have been limited by inactivity of the mitochondrial fraction and have so far led to a limiting rate of photosynthesis which is only 1 % of that found in the intact leaf. The reason for this reduction in photosynthetic ability upon disrupting the cell and isolating the chloroplasts is not known, but the fact that addition of sap increased the photosynthetic rate more than three times offers some opportunity for further experimentation on this problem.

Addition: Since the completion of the manuscript, Gibbs and Cynkin (1958 a) have reported a CO_2 fixation rate of $0.033 \mu\text{mole/min.} \cdot \text{mg. chloro-}$

phyll by spinach chloroplasts. On this basis, the maximum rate of CO_2 fixed by intact spinach chloroplasts is only about 1 % that of the maximum CO_2 fixation rate by intact *Chlorella*.

Summary

Cellular constituents (chloroplasts, sap and mitochondria), separated from spinach leaves, were allowed to photosynthesize in the presence of radioactive bicarbonate. The radiocarbon fixed by the chloroplasts was distributed mainly in the phosphate esters while the radiocarbon fixed by the sap was located mostly in the amino acids. The mitochondrial fraction fixed very little radioactive carbon dioxide. The rate of carbon dioxide fixation by the recombined mixture of sap and chloroplasts was greater than the sum of the separated components. The distribution pattern of the fixed radiocarbon of the recombined mixture, however, was qualitatively similar to a combination of the radiocarbon distribution of the separated chloroplasts and sap. The results of experiments carried out with these cellular constituents in the dark and in the presence of other substrates, labelled and unlabelled, are discussed.

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Effect of Pectinase and Cellulase Preparations on the Growth and Development of Root Hairs

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Introduction

Recently, Cormack (1955, 1956) and Ekdahl (1957) have used commercial preparations of pectinase and cellulase as analytical tools in testing their diametrically opposed hypotheses concerning the cell wall structure and development of root hairs. Cormack (1935, 1949) has maintained that the root hair cell wall is composed of two distinct layers; an outer one of pectic substances and an inner layer of cellulose. He believes that the hardening of the cell wall behind the plastic root hair tip is brought about by the reaction between calcium ions in the external medium and pectic acid in the outer layer of the wall. Ekdahl (1953) in rejecting the above hypotheses, suggests that the root hair cell wall is composed of a framework of cellulose with pectic substances acting as an incrusting cement throughout the structure. He believes that the plasticity of the root hair tip is maintained by the action of pectic and cellulosic enzymes and that the hardening of the root hair cell wall behind the tip is due to the incorporation of cellulose microfibrils. The microfibrils are deposited at random initially, but become oriented more or less parallel to the longitudinal axis of the root hair as growth continues.

It is well known that commercial preparations of enzymes may have considerable quantities of impurities. In view of the fact that neither Ekdahl nor Cormack took special pains to purify the enzymes used as analytical tools in testing their hypotheses, a further study of this problem was in order.

In the present study, primary roots of redtop grass (*Agrostis alba* L.) seed-

lings were subjected to pectinase, autoclaved pectinase, cellulase, and autoclaved cellulase preparations in standing solutions. Root hair elongation, change in diameter, and resumption of growth following treatment were studied. Results of the present study show that in redtop grass roots some of the effects which both Cormack and Ekdahl have attributed to specific enzyme action and which they maintain support their individual hypotheses can be duplicated by these preparations after inactivation of the enzymes by heat. Furthermore, the multienzymatic nature of these preparations precludes the possibility of attributing their effects to the action of a specific enzyme.

Materials and Methods

Redtop grass caryopses were sown on moist lens paper in a culture chamber and allowed to develop until the primary root was 10 to 15 mm. in length. The culture chamber (Goodwin and Avers 1956) was constructed by cementing thin strips of glass to a 50×75 mm.×1 mm. microscope slide and cementing a 35×60 mm. cover glass to the glass strips to form a water-tight compartment open at the top. After adding lens paper strips and a small amount of culture solution, the caryopses were sown along the moist lens paper at the top of the chamber. The chamber was then stored in a slightly inclined position so that the emerging roots would grow down along the cover glass, thereby facilitating examination with the microscope. Four days later, the chamber was drained and refilled to just below the caryopses with fresh culture solution. After a 2 to 3 hour wait, the chambers were transferred to the stage of a horizontal microscope. Normally, one root was selected for study, sketched, and 5 to 8 root hairs selected for measurement and numbered on this sketch. The hairs and primary root were measured at 30 minute intervals for a two hour control period. The solution was then drained, replaced with the test solution and measurements recorded every 30 minutes for an additional 2 hour period. Measurements and observations were made at infrequent intervals over the next 24 hours. The hairs and primary root were measured by means of an ocular micrometer at total magnifications of 645 and 150×, respectively. This constituted a single experiment. Each experiment was replicated at least 3 times. In the experiments in which root hair diameter was determined, measurements were made with an ocular micrometer at a total magnification of 645×.

The ordinary culture solution used throughout the study has the following composition:

	Concentration in millimoles per liter
KNO ₃	0.6
Ca(NO ₃) ₂	1.5
MgSO ₄	1.0
Succinic acid	5.0
NaOH	10.0

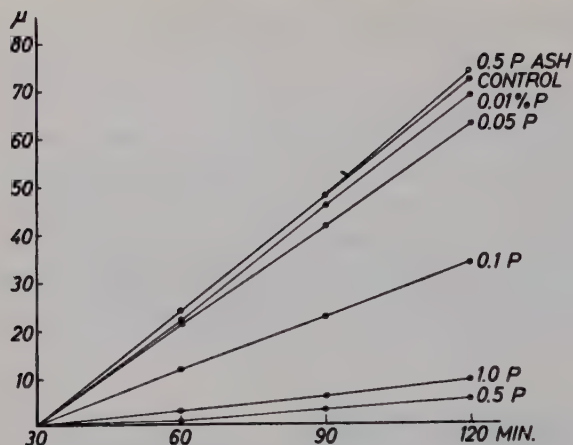


Figure 1. Effect of different concentrations of pectinase and ash from 0.5 % pectinase on root hair elongation. On the abscissa time in minutes after treatment. On the ordinate elongation in μ .

The pH of the solution was adjusted to pH 5.0—5.1. All test solutions were prepared from double glass-distilled water. The Pectinase preparation was obtained from Nutritional Biochemicals Corporation; Cellulase was from Delta Chemical Co. These preparations were not purified in any way before use except as indicated below; therefore, it should be borne in mind that the terms "pectinase" and "cellulase" as used in this paper refer to these preparations and do not imply that they have only a single enzymatic activity. The enzyme preparations were made up in the ordinary culture solution detailed above, centrifuged at low speed to remove the diatomaceous earth filler and other debris, aerated vigorously, adjusted to pH 5.0—5.1, and allowed to come to room temperature before use. Inactivation of the enzymes present in the pectinase and cellulase preparations was accomplished by autoclaving the solution for 5 minutes at 15 lbs. pressure, *i.e.* at a temperature of 121 degrees C.

Results

Pectinase Experiments

Both Cormack (1955, 1956) and Ekdahl (1957) reported that pectinase inhibited root hair elongation. This was also found to be true in the present experiments with root hairs of redtop grass seedlings (Figure 1). 0.1 % pectinase reduces elongation by about 50 % during the period 0.5 to 2.0 hours after treatment and 0.5 % pectinase reduces elongation to less than 10 % of the controls. Ashing such pectinase preparations destroys their ability to inhibit root hair elongation.

The effect of autoclaved pectinase preparations over the same concentration range was also studied. A comparison of root hair elongation rates in pectinase versus autoclaved pectinase preparations for the period 0.5 to 2.0 hours after treatment is given in Table 1. The rate of root hair elongation

Table 1. *Elongation of root hairs in pectinase preparations.* Values represent the elongation rate in the period 0.5 to 2.0 hours after treatment expressed as a percent of the control rate.

Preparation	Enzyme preparation concentration in percent				
	0.01	0.05	0.10	0.50	1.00
Pectinase	108 \pm 4.5	97 \pm 7.2	50 \pm 8.6	9 \pm 6.3	16 \pm 7.0 ¹
Autoclaved pectinase	109 \pm 6.2	38 \pm 9.3	2 \pm 2.0	0	0
Difference between means	1	59	48	9	16
2 \times Std. error of diff.	15.3	23.6	17.6	12.5	14.0

¹ Standard error of the mean.

decreases with increasing concentrations of pectinase. However, this is also true in the autoclaved pectinase preparations. In fact, at the 0.05 and 0.1 % levels, autoclaved pectinase causes a significantly greater reduction in elongation rate than does pectinase. 0.1 % autoclaved pectinase causes almost complete inhibition of elongation whereas the same concentration of pectinase only reduces the rate to 50 % of the control.

Neither Cormack nor Ekdahl studied the recovery of individual root hairs following treatment. However, our early observations indicated that this would be of interest. We recorded the number of hairs which either continued to elongate following treatment or which resumed growth in the period 2 to 24 hours after treatment (Table 2). At concentrations of 0.05 % or lower, there is no significant effect of either pectinase or autoclaved pectinase on growth of root hairs following treatment. Only one-fifth of the hairs recover in 0.1 % autoclaved pectinase and none recover at higher concentrations. In the pectinase preparations, two-thirds of the hairs recover even at the highest concentrations used.

Cellulase experiments

Ekdahl (1957) concluded that the decreased maximum root hair length which he observed in both Brassica and wheat roots subjected to a cellulase preparation supported his theory that the action of pectic and cellulosic en-

Table 2. *Resumption of growth or continued elongation of root hairs 2 to 24 hours after treatment with pectinase preparations.* The values represent the percent of the total number of hairs being measured which were elongating or resumed growth during the above period.

Preparation	Enzyme preparation concentration in percent				
	0.01	0.05	0.10	0.50	1.00
Pectinase	96	100	87	71	67
Autoclaved pectinase	100	91	19	0	0

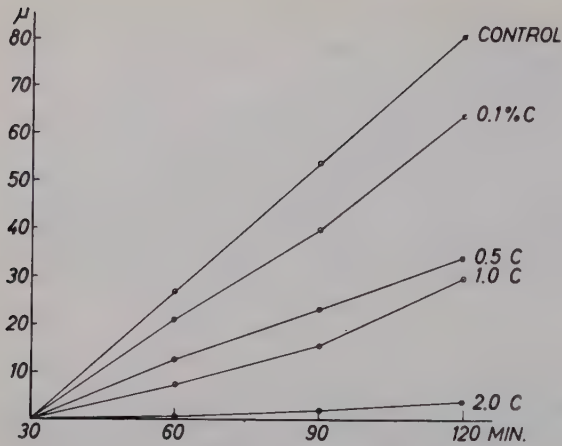


Figure 2. Effect of different concentrations of cellulase on root hair elongation. As Figure 1.

zymes in the root hair tip was responsible for its continued plasticity. We studied the effect of the same preparation on the elongation of root hairs in redtop seedlings. In the period 0.5 to 2.0 hours after treatment with cellulase, increasing concentrations result in increasing inhibition of root hair elongation (Figure 2). 0.5 to 1.0 % cellulase is required to reduce elongation by about 50 %. There is still some growth even in 2.0 % cellulase. There is a definite increase in rate of elongation during the last 30 minutes of this period at all concentrations. A comparison of this figure with figure 1 suggests that commercial pectinase is more effective than commercial cellulase at equal concentrations in inhibiting root hair elongation. Also, recovery during the period 1.5 to 2.0 hours after treatment is quite marked in cellulase at all concentrations, but is not observed in pectinase.

The effect of the same concentrations of autoclaved cellulase preparations on root hair elongation was determined. A comparison of root hair elongation rates in cellulase versus autoclaved cellulase preparations for the period 0.5 to 2.0 hours after treatment is given in Table 3. In the range studied, increas-

Table 3. Elongation of root hairs in cellulase preparations. Values represent the elongation rate in the period 0.5 to 2.0 hours after treatment expressed as a percent of the control rate.

Preparation	Enzyme preparation concentration in percent			
	0.1	0.5	1.0	2.0
Cellulase.....	83 ± 5.1	50 ± 4.5	44 ± 9.0	4 ± 1.5
Autoclaved cellulase	90 ± 5.6	31 ± 6.8	10 ± 4.2	3 ± 1.1
Difference between means	7	19	34	1
2 × Std. error of diff.	15.0	16.2	18.7	3.6

¹ Standard error of the mean.

Table 4. *Resumption of growth or continued elongation of root hairs 2 to 24 hours after treatment with cellulase preparations.* The values represent the percent of the total number of hairs being measured which were elongating or resumed growth during the above period.

Preparations	Enzyme preparation concentration in percent			
	0.1	0.5	1.0	2.0
Cellulase.....	100	74	75	76
Autoclaved cellulase	94	65	44	63

ing the concentration of either cellulase or autoclaved cellulase causes an increasing inhibition of root hair elongation. At the 2.0 % level, inhibition is so severe that there is little difference in response of root hairs to the two preparations, and at the 0.1 % level inhibition is so slight in both preparations that there is only a slight difference in degree of inhibition. However, at the intermediate levels of 0.5 and 1.0 %, autoclaved cellulase inhibits root hair elongation to a significantly greater extent than does the unautoclaved enzyme.

Continuance or resumption of growth of root hairs in the period 0.5 to 2.0 hours after treatment with various concentrations of cellulase or autoclaved cellulase was also recorded. These results, expressed as percentages of the total number of hairs being measured, are given in Table 4. A slightly greater percentage of hairs recover in cellulase than in autoclaved cellulase at all concentrations used. It should be pointed out that although 44 % of the hairs recover under the most adverse conditions in the cellulase preparations, only one-fifth of the hairs recover in 0.1 % autoclaved pectinase (Table 2) and none recovered at higher concentrations.

Ekdahl (1957) maintains that the increase in root hair diameter in rape and wheat seedlings following treatment with pectinase, pectinol, and cellulase supports his theory that pectic and cellulosic enzymes maintain the plasticity of the root hair tip. Root hairs of redtop grass seedlings also increase in diameter when treated with cellulase. Although Ekdahl only measured hairs

Table 5. *Effect of active and autoclaved cellulase preparations on the diameter of root hairs elongating at the time of treatment and those which originate in the test solution.*

Preparation	Diameter in microns		Diff. between means	2 \times Std. error of means	Treat/control in %
	Control	Treated			
0.5 % Cellulase	8.87	12.81	3.94	0.35	144
0.5 % Autoclaved cellulase	8.07	10.69	2.62	0.26	132
0.1 % Cellulase	8.09	10.15	2.05	0.52	125
0.1 % Autoclaved cellulase	7.58	10.12	2.55	0.61	134

which developed in the enzyme preparations in arriving at the increased diameter figures, we find that root hairs growing at the time of treatment with cellulase bulge and continue to grow at an increased diameter. We also find that the diameter of hairs which originate in the enzyme preparations are greater than the diameter of hairs developed before treatment. These results, as well as results obtained by subjecting roots to equal concentrations of autoclaved cellulase, are shown in Table 5. Both cellulase and autoclaved cellulase preparations at the 0.1 and 0.5 % levels cause significant increases in root hair diameters. A comparison of the figures in the last column, in which the root hair diameters following treatment are expressed as percentages of the control diameters, reveals that the increase in root hair diameter cannot be attributed to enzyme action.

Discussion

In the present work, it has been demonstrated that some of the effects of commercial preparations of pectinase and cellulase on root hairs of redtop grass seedlings are not due to enzymatic activity. As has been found by other workers for other species, both pectinase and cellulase inhibit root hair elongation. However, autoclaved pectinase and cellulase preparations also inhibit root hair elongation. In fact, in some instances, the autoclaved preparations are even more inhibitory than the untreated preparations. Inhibition is not caused by the action of inorganic components of the preparations.

From these results, it is suggested that earlier workers are not justified in attributing the inhibition of elongation observed in root hairs subjected to commercial pectinase and cellulase preparations to the activity of enzymes in the preparations.

The resumption of root hair growth following treatment with pectinase and cellulase preparations provides additional insight into the possible role of the enzymes present. It appears that there are non-enzymatic components of these preparations which severely inhibit root hair elongation, but if the enzymes normally present in the preparation are in an active form, this inhibition can be largely overcome.

There was a highly significant increase in root hair diameter in redtop seedlings subjected to dilute solutions of cellulase. Hairs which were developing at the time of treatment, as well as those which originated in the treatment solution, exhibited this marked increase in diameter. However, the autoclaved cellulase preparations also caused an increased root hair diameter

of the same magnitude as that induced by cellulase. These results make it clear that the increase in root hair diameter is not mediated by the added cellulase.

Crude pectinase and cellulase preparations commercially available contain many enzymes in addition to those suggested by the name. For example, the fungal extract designated as pectinase contains, in addition to pectin methyl-esterase and polygalacturonase, enzymes which hydrolyze sucrose and inulin (Pigman 1943), starch and maltose (Fish and Dustman 1945), sodium salt of carboxymethylcellulose and gum tragacanth (Lineweaver, Jang, and Jansen 1949). Siu (1951) notes that commercial preparations, presumably containing cellulolytic enzymes, together with enzymes degrading hemicellulose, fats, and proteins, have been sold as digestive aids. With these facts in mind, it is difficult to understand how these preparations can be used as analytical tools. One certainly is not justified in claiming that specific enzymes in these preparations produce the observed effects.

Summary

Roots of redtop grass seedlings were subjected to dilute solutions of commercial preparations of pectinase and cellulase. The effects of these treatments on root hair elongation and diameter increase were similar to effects reported for other species by other workers. However, it was found that autoclaved preparations induced the same changes and, in fact, were even more effective. Both pectinase and autoclaved pectinase preparations inhibited root hair elongation; cellulase and autoclaved cellulase inhibited root hair elongation and caused an increase in root hair diameter. Resumption of growth in the period two to twenty-four hours after treatment was much greater in active preparations than in autoclaved ones. It is clear that some of the effects of pectinase and cellulase preparations reported by previous workers are not enzyme-mediated. Furthermore, the multienzymatic nature of these preparations has not been recognized by these workers who have used them to test divergent hypotheses concerning root hair growth and cell wall composition.

It should be emphasized that commercial sources of pectic and cellulosic enzymes are unsuitable for testing hypotheses concerning root hair cell wall composition and growth of root hairs.

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Comparative Evolutionary Aspects of Polyglucoside Synthesizing Enzymes

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The synthesis of polysaccharides from glucose-1-phosphate involves a system composed of two enzymes, *phosphorylase* and *branching* (or "Q") *enzyme*. In brief, phosphorylase synthesizes α 1 : 4-glucosidic linkages thereby building up *linear* maltodextrins. Q enzyme establishes α 1 : 6-glucosidic linkages by fragmenting and altering the linear maltodextrins resulting from phosphorylase action, and hence, "*branching*" these linear polymers.

The resulting sugars (polyglucosides) show a spectrum of branching ranging from the relatively unbranched *amylose* molecule through the branched *amylopectins* to the highly branched *glycogens*. A comparison of the type of polyglucoside synthesized by different evolutionary groups illustrates this variation. For example, the blue-green alga, *Oscillatoria princeps*, forms a glycogen-like polyglucoside (Fredrick 1951, 1953). The phytomonad, *Polytomella coeca*, synthesizes an amylopectin-like sugar (Barker *et al.* 1953). The polyglucoside present in higher plants such as the pea and the potato is starch, a mixture of amylopectin and amylose (Hanes 1940 a, 1940 b, Green and Stumpf 1942). In animals, the storage polyglucoside is invariably, glycogen (Cori and Cori 1945).

The polyglucoside synthesized by a particular group is, within certain limits (Schlamowitz 1951), rather specific to the group. For example, oyster glycogen is very highly branched and quite different from rabbit glycogen (Schlamowitz 1951) or phytoglycogen (Fredrick 1953). These are, of course, widely divergent evolutionary groups, but the same variation in polyglucoside branching is exhibited by closely related organisms within a subphylum. In

the algae, various degrees of branching in the polyglucosides progressing from the Cyanophyceae through the Chlorophyceae have been demonstrated (Fredrick 1952b). There appears to be evidence too, that in this group the type of polyglucoside is related to the evolutionary stage of the *plastid* (Yin 1948, Weier 1953); the more primitive algae (the blue-greens) with no apparent definitive plastids give rise to more highly branched polyglucosides than do the green algae where the plastids are developed, definitive structures. Hence, it seems more than coincidental that with the evolving of this highly important organelle in the plant cell, there should be a corresponding *decrease* in the degree of branching of the polyglucoside produced, with a tendency toward the formation of relatively unbranched α 1 : 4-glucosidic linked linear maltodextrins in the higher groups.

In order to discuss the interrelations of phosphorylase and Q enzyme from a comparative evolutionary standpoint, it is necessary that the mode of action of these enzymes be understood. Phosphorylase will *simultaneously* add glucose residues from the substrate, glucose-1-phosphate, in α 1 : 4 linkages to a "primer". The size of this "primer" has been a subject for speculation. There is evidence that it may be a maltodextrin of short chain-length (such as maltotriose), of long chain-length (such as amylose), branched (such as amylopectin or glycogen), or perhaps not necessary at all (Fredrick 1951, Fredrick and Mulligan 1955, Fredrick 1958). In this latter case, S-shaped reaction curves result which are highly suggestive that phosphorylase unites glucose-1-phosphate residues, slowly building up its own "primer", and then rapidly adding to it until a fair-sized linear maltodextrin results.

Q enzyme (or branching enzyme) has been shown to have two specific actions. First there is the apparently *random* scission of the linear maltodextrin synthesized by the phosphorylase (Peat *et al.* 1953, Barker *et al.* 1953, Nussenbaum and Hassid 1952), and then, an establishment of α 1 : 6-glucosidic linkages between the fragments of the maltodextrin. The limiting factors are entirely dependent upon the synthesis of substrate for Q enzyme action by the phosphorylase. This, of course, brings up the question as to the minimum size of the linear maltodextrin which can act as substrate for Q enzyme. It was previously thought that long chain-lengths of about 116 units were required (Nussenbaum and Hassid 1952), but later work showed that a 42-unit and a 28-unit maltodextrin were also branched by Q-enzyme (Peat *et al.* 1953, Barker *et al.* 1953). Recently, the minimum chain-length necessary for *Oscillatoria princeps* Q enzyme has been reported to be one of 6 units (or maltohexaose) (Fredrick and Mulligan 1955, Fredrick 1955). But, in all cases, a certain minimum polymer of α 1 : 4-glucosidic linked units is necessary for the action of this enzyme. This polymer is supplied by the action of the phosphorylase of the mixture.

Table 1. *Comparison of the physico-chemical properties of phosphorylases from various sources.*

Source	Electrophoretic mobility ("μ")	Michaelis-menten constant	Isoelectric point ("pI")	pH optima
<i>O. princeps</i> , <i>n</i>	-3.4×10^{-5} cm ² /volt. sec. ⁽¹⁾	8×10^{-5} M. ⁽²⁾	5.8 (1)	6.8 (3)
<i>O. princeps</i> , <i>LTV</i>	-3.6×10^{-5} (1)	8×10^{-5} (2)	5.9 (1)	6.8 (3)
Rice	—	—	5.0 (4)	6.6 (5)
Potato	—	—	5.0 (6)	6.5 (5)
Rabbit	-3.1×10^{-5} (7)	8×10^{-5} (8)	—	6.8 (7)

References:

- ¹ Fredrick 1956.
- ² Fredrick and Mulligan 1955.
- ³ Fredrick 1959.
- ⁴ Aimi and Murakami 1954.
- ⁵ Aimi et al 1956.
- ⁶ Aimi and Nishio 1956.
- ⁷ Krebs and Fischer 1956.
- ⁸ Cori, Cori and Green 1943.

Phosphorylases

Do the phosphorylases of different groups differ in themselves? Since phosphorylase is undoubtedly the limiting factor in Q enzyme activity, it would seem reasonable to assume that the differences existing in the various polyglucosides reflect a molecular difference in the physical-chemical constitution of this enzyme from group to group.

Table 1 contains a comparative list of the physical-chemical properties of phosphorylases from various sources. From a perusal of this table, even though the data have been compiled from different sources with no attempt at standardization, it is nonetheless apparent that a more than casual relationship exists among these phosphorylases.

This "sameness" is particularly important in the closely related *n* and *LTV* *Oscillatoria princeps*, where although the enzymes seem to be physically and chemically identical, the polyglucosides synthesized are quite different (Fredrick 1953, Fredrick and Mancini 1955, Fredrick 1956). Also, in connection with the identity of these phosphorylases, it should be pointed out that all of the enzymes listed in this table are capable of being "primed" by any of the polyglucosides formed by the other phosphorylases. This would seem to indicate a sort of phylogenetic continuity on their part. Attention should also be called to the fact that even animal phosphorylase is capable of synthesizing amylose-like sugars (Bear and Cori 1941).

Q enzymes

Since no apparent physico-chemical differences are present in the phosphorylases discussed, it may be that such differences are present in the intimately associated enzyme, the Q enzyme. In *Oscillatoria princeps*, both the *n* and *LTV* mutant have branching enzymes which are identical in physico-chemical properties (Fredrick and Mancini 1955, Fredrick 1956). Although detailed studies of the physico-chemical properties of Q enzymes from different organisms are not available, their action mechanisms indicate an extremely close similarity.

The observation that β -limit dextrins persisted after β -amylase action on the polyglucoside synthesized by the Q enzyme of potato acting on a 28-unit dextrin, served to lower still further the requirement for the minimum chain-length originally thought to be necessary for Q enzyme action (Peat *et al.* 1953). Recent work on the Q enzyme of *Oscillatoria princeps* indicates that the minimum chain-length needed is probably of the order of maltohexaose (a six unit linear maltosaccharide) (Fredrick and Mulligan 1955). In *Polytomella coeca*, the Q enzyme is activated by shorter linear maltosaccharides of from two to twenty units (Barker *et al.* 1953). It would seem that Q enzymes from these different sources have similar modes of action. Even more so, when one considers that these different Q enzymes can all act on each other's minimum substrates (Barker *et al.* 1953).

Therefore, since the phosphorylases are not different from group to group (Cf. Table 1), and since the Q enzymes also appear to be the same, the only logical explanation for the different polyglucosides synthesized by these similar, if not identical, enzymes must be in the relative concentrations or *activities* of phosphorylase and Q enzyme in the organism, or in a mixture of these enzymes in an isolated system (Fredrick 1952, 1953).

The Q/P ratio

It was suggested by Schlamowitz (1951) that the differences in the degree of branching of various polyglucosides of animal origin might be accounted for by a decrease in the concentration of the branching enzyme (or Q enzyme). This explanation was also used for the differences in the degree of branching of the two phytoglycogens isolated from *n* and *LTV* strains of the blue-green alga, *Oscillatoria princeps* (Fredrick 1952, 1953). In either instance, it is obvious that *a decrease in concentration or activity of the Q enzyme component of the ratio is the same as an increase in concentration or activity of the phosphorylase (P) component*. How then, are these changes in the ratio brought about?

It is, of course, not necessary to increase or decrease the concentration of the enzymes at all to alter their *activities*. The same effect can result from the partial *inhibition* of one of the enzyme pair. It has been shown that naturally occurring *amino acids* may act as inhibitors of animal phosphorylase (Fredrick *et al.* 1951). The mechanism of this type of inhibition has been demonstrated in *Oscillatoria* phosphorylase to be one of *chelation* (Fredrick 1957). A further report indicates that the effect of chelating agents on algal phosphorylase is such that they transform *active enzyme* to *inactive protein* by removing the *manganese* essential for phosphorylase activity (Fredrick 1951, 1958). Manganese has been implicated in phosphorylase activity in yeast (Kiessling 1939), and was thought to be necessary for animal phosphorylase action (Cori and Cori 1945) together with adenylic acid. It has been subsequently shown that animal phosphorylase exists in two forms, *a* and *b* (Keller and Cori 1955), and that the inactive *b* form can be transformed to the active *a* form in the presence of manganese ion (Krebs and Fischer 1956). Perhaps both the algal and animal mechanisms with regard to the activity of phosphorylase are similar; but, nevertheless, it is obvious that any mechanism resulting in a decrease or increase in the activity of phosphorylase can "control" the Q/P ratio and hence, determine the branching characteristics of the polyglucoside ultimately synthesized.

If the Q/P ratio is such that the *P* component is greater, the resulting sugar will show *less* branching, and therefore be more closely related to amylose (Schlamowitz 1951, Nussenbaum and Hassid 1952, Peat *et al.* 1953, Fredrick 1953). If, on the other hand, Q enzyme activity predominates in the ratio, then the resulting sugar will be more highly branched such as glycogen.

Possible mechanism for evolution based on the Q/P ratio

From this discussion, it seems apparent that once the cytogenetic elaboration of each enzyme (phosphorylase and Q enzyme) has taken place, the adaptability of the interaction of the two enzymes in forming the "right" kind of polyglucoside demanded by the organism as a result of its particular environment, can be accomplished via genes which act as suggested by Goldschmidt (1938) to control *rates* of physiological processes. These genes would control the relative activities of phosphorylase (and Q enzyme?) much in the way that "shape" genes control that attribute in plants (Sinnott 1958), possibly through the intervention of naturally occurring chelating agents such as the amino acids.

Studies now in progress reveal that this mechanism is operative in *Oscillatoria princeps*, and that the differences in the branching characteristics of

the *n* and *LTV* types of *Oscillatoria* sugars are the result of the presence of a group of natural inhibitors of a chelating nature in *n* *Oscillatoria*, and their practically complete absence in *LTV* *Oscillatoria*. These chelating inhibitors appear to be of amino acid nature (Fredrick 1959).

It is possible then, that the presence of glycogen-like polymers in *Oscillatoria princeps* is due to the effect of these inhibitors on the phosphorylase component of the Q/P ratio, and that there is a gradual elimination of phosphorylase-inactivating substances as one progresses from the Cyanophyceae to the Chlorophyceae where obviously full activity of the phosphorylase component is indicated by the synthesis in green algae of amylose-like, relatively unbranched structures. It is suggested that this may be a *modus operandi* for the adaptation of these enzymes in plants, and possibly a path of evolution intimately associated with that of the plastid.

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Changes in Microsomal Ribonucleoproteins in the Time Course of the Germination Stage as Revealed by Electrophoresis

By

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It has been reported that at the outset of germination of *Vigna sesquipedalis* seeds the decrease in ribonucleic acid (RNA) content in a pair of cotyledons is precisely balanced with the increase in RNA in the seedling axis (plumules + epi- and hypocotyls + radicle), whereas in the isolated cotyledons from the growing tissues no change in the RNA level takes place (Oota and Osawa, 1). On the other hand, in the hypocotyl at the middle of the germination stage (the definition of the germination stage has been given by Oota *et al.*) when the supply of cotyledonous reserves including RNA has nearly been exhausted, net synthesis of protein ceases entirely to proceed and a marked drop in the RNA level occurs, and the drop is almost wholly recovered as a simultaneous sharp rise in the RNA level of the epicotyl (Oota *et al.*). In contrast to a considerable growth of the hypocotyl in the first half of the germination stage, no significant growth of the epicotyl initiates until the mid-germination stage, *i.e.*, typical epigeal germination. If the epicotyl (together with the cotyledons and the plumules) is then removed, the RNA level of the hypocotyl is maintained almost unchanged thereafter throughout the latter half of the germination stage (Oota and Osawa, unpublished, *cf.* Oota). These facts appear to be hardly explainable without assuming ready migration of intact or nearly intact RNA molecules in the germ tissues in question.

Participation of microsomal RNA in protein synthesis is widely accepted. It is believed to be also the case for the bean hypocotyl where the rate of pro-

tein synthesis is in a linear relationship with the RNA content of microsome fraction (Oota and Osawa, 2). Noteworthy is that in the hypocotyl in the latter half of the germination stage the RNA level of microsome fraction drops, while that of soluble cytoplasm fraction tends to rise, the protein level of either fraction being altered little (Oota and Osawa, 2, Oota).

From these findings a working hypothesis has been introduced that RNA in the bean germ tissues would involve at least two types, *i.e.* *functional* and *transportable RNA*-s. The former may be microsomal ribonucleoprotein participating directly in protein synthesis. As the age of the tissues advances, the nucleic acid will at least partially dissociate from the protein moiety of *functional RNA* to be released into soluble cytoplasm. It can now be *transportable RNA* which is to be transferred to younger or vividly growing tissues and there to be reconverted into *functional RNA*. *Transportable RNA* will assumingly be intact or nearly intact molecules of RNA (Oota).

As the first step of examination of the hypothesis, electrophoretic analyses were undertaken of cytoplasmic nucleoproteins extracted from microsome fractions of the hypocotyls and the cotyledons of germinating bean seeds. The results obtained appear to illustrate conversion steps from *functional RNA* to *transportable RNA*, as reported in the present paper.

Materials and Methods

Extraction of microsomal nucleoproteins. The seeds of *Vigna sesquipedalis* stored in a dark desiccator for about two years after harvest were used. The seeds were germinated in the dark at 30°C. and germ tissues to be examined were separated in a similar way as previously described (Oota *et al.*). As the 0 day-old materials pre-soaked seeds just before being sown were employed. All ensuing operations including electrophoretic ones were conducted in a cold room at about 4°C. The isolated hypocotyls or cotyledons were rinsed repeatedly with ice-chilled distilled water and ground thoroughly in a porcelain mortar with adequate amounts of sucrose (the final concentration: 0.25 *M*) and sea sand. The mortar, pestle and sucrose solution were chilled in the cold room before use. The homogenates were squeezed through cotton cloth. After larger particles were discarded with a Servall centrifuge (Model SS-1) at 10,000×*g* for 20 min., microsome fraction was sedimented by centrifugation at 90,000×*g* for 90 min. using a Spinco centrifuge (Model L). The supernatant was discarded as completely as possible. The brownish yellow pellets obtained were homogenized mildly in a glass homogenizer with a small amount of phosphate buffer (pH 7.1, 0.1 *M*) (*cf.* Takata and Osawa). After being allowed to stand for 20 min. the suspension was centrifuged at 18,400×*g* for 30 min. with the Servall centrifuge. The slightly turbid supernatant obtained (pH 7.1-fraction as called by Osawa *et al.*) were immediately used for electrophoresis examination. As shown in Table 1, the above-described buffer treatment can "extract" the bulk of RNA, about 85 per cent of protein and one third of phosphatid of the microsome fractions.

NORMAL PLANTS

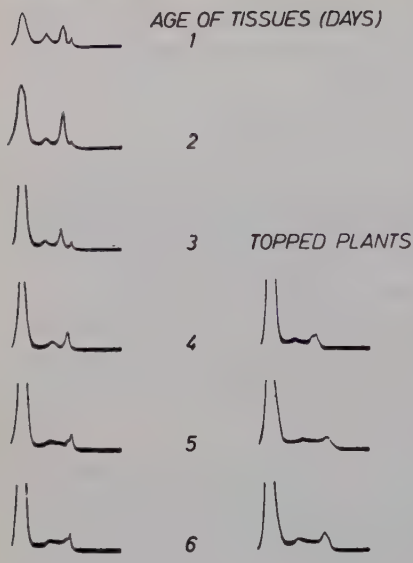


Figure 1. Descending electrophoretic patterns of pH 7.1-fractions prepared from etiolated bean hypocotyls of various ages. Left: Normal plants; 2 ml. of pH 7.1-fractions equivalent to 154, 92, 96, 84, 73 and 70 hypocotyls for 1, 2, 3, 4, 5 and 6 day-old samples used respectively. Right: Topped plants; 2 ml. of pH 7.1-fractions equivalent to 63, 70 and 74 hypocotyls for 4, 5 and 6 day-old samples used respectively. All with phosphate buffer, pH 7.1, 0.1 *M*; field strength, 1.5 v./cm.; 60 min. of migration. For further details see the text.

Electrophoretic analyses. Electrophoretic runs were conducted in the extraction buffer (pH 7.1, 0.1 *M*) in a 2 ml.-cell of a Tiselius-type apparatus (Hitachi, Model HT-B). An aliquot of the pH 7.1-fractions was placed in the cell without previous dialysis. Ascending and descending limbs yielding essentially similar patterns, more clear-cut separation of the components was achieved in the latter. Photographs were taken twice, at 60 and 100 min. of migration. The samples prepared on separate occasions from tissues of comparable ages gave fairly consistent patterns. For the sake of convenience, the conductivity of the extraction buffer, in place of those of pH 7.1-fractions, was used throughout in the calculation of mobilities (*u*).

Results

Hypocotyls. Typical descending patterns for the pH 7.1-fractions of the hypocotyls excised from the normally grown seedlings are shown in Figure 1. A remarkable stationary boundary may be attributable, in all likelihood, to sucrose contaminant. In the 1 to 3 day-old tissues, or in the first half of the germination stage, the pH 7.1-fraction contains three components, which are arbitrarily named Components I ($u=-6$), II (-10) and III (-12) in order of their descending mobilities. In view of their high mobilities all of these components may be ascribed to ribonucleoproteins, and the greater the mobility the higher will be the ribonucleic acid concentration of the component. Preliminary chemical and spectral analyses made on isolated com-

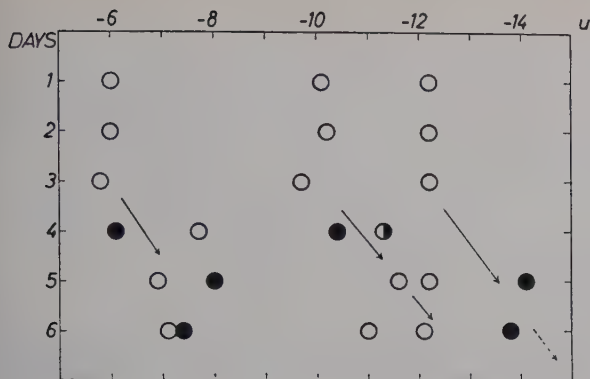


Figure 2.

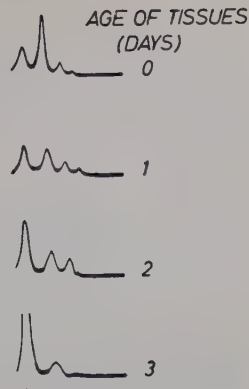


Figure 3.

Figure 2. *Descending mobilities of electrophoretic components of pH 7.1-fractions prepared from etiolated bean hypocotyls of various ages. Mobilities computed on the results shown in Figure 1. ○: normal plants, ●: topped plants. On the abscissa mobility (u), $\text{cm}^2/\text{sec. v.} \times 10^{-5}$, on the ordinate age of tissues, days.*

Figure 3. *Descending electrophoretic patterns of pH 7.1-fractions prepared from etiolated bean cotyledons of various ages. 2 ml. of pH 7.1-fractions equivalent to 11, 39, 50 and 40 pairs of cotyledons for 0, 1, 2 and 3 day-old samples used respectively. All with phosphate buffer, pH 7.1, 0.1 M; field strength, 1.5 v./cm.; 60 min. of migration. For further details see the text.*

ponents have evidenced this view, and furthermore suggested that the component of the highest mobility may be ribonucleic acid free from protein. The relative concentration of Component II, as estimated by computing the area under the scanning peak, is always the highest among others.

As the mid-germination stage is passed, a change or disturbance begins to appear in the electrophoretic pattern which has hitherto been fairly steady within the range of experimental error (*cf.* Figure 2). Thus the major component (II) disappears and a new component of higher mobility ($u = -11.5$) arises instead. It merits attention that the mobility of this new component approximates to that of Component III. Component III, after a temporal but complete disappearance in the 4 day-old tissues, restores its quantity. The slowest moving peak (Component I) is also replaced by a peak of slightly higher mobility (*ca.* -7).

Figures 1 and 2 also include the results of electrophoretic analyses conducted for pH 7.1-fractions prepared from the topped hypocotyls. The 3 day-old plants were topped at the point of cotyledon attachment by a well rinsed razor blade under dim room light. The samples were collected at daily interval for 3 consecutive days and the hypocotyls whose tops were somewhat swollen were isolated to be used for the preparation of pH 7.1-fractions. As seen in these figures, marked effects of topping at the

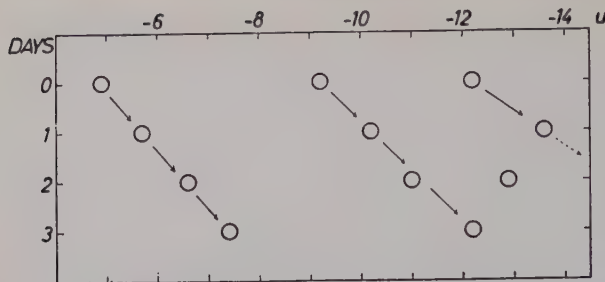


Figure 4. Descending mobilities of electrophoretic components of pH 7.1-fractions prepared from etiolated bean cotyledons of various ages. Mobilities computed on the results shown in Figure 3. As figure 2.

mid-germination stage are that both Components II and III disappear rather rapidly and that a significant amount of a new component substance of very high mobility (ca. -14 , designated Component IV) is accumulated.

Cotyledons. Only the first half of the germination stage was investigated for the cotyledons, since by the end of this period the role of the tissues as "primary storehouse of the germs" (see the discussion) is considered to be nearly finished (Oota *et al.*). In this case also, as seen in Figures 3 and 4, three well-defined peaks are detected at the outset of germination besides 'sucrose boundary', to which we shall refer Components I' ($u = -5$), II' (-9) and III' (-12). Our unpublished investigation has shown that Components I' and II' and Component III' may be ribonucleoproteins and free ribonucleic acid, respectively. The identity of the cotyledon components with the corresponding hypocotyl ones, i.e., I' *vs.* I, II' *vs.* II, III' *vs.* III and, further, the below-described IV' *vs.* IV will be asked elsewhere. It is only noted here that all electrophoretic components so far examined well agree with each other as regards their nucleotide compositions (Oota and Takata, unpublished).

An obvious change occurs in the electrophoretic pattern with the age of the tissues (*cf.* Figure 4). Thus, as indicated by the arrows in the figure, the mobility of Component II' seems to rise until it finally comes to the original mobility of Component III', which in turn is likely converted to a new component of higher mobility (Component IV', $u = -13.5$) as revealed for the 1 day-old samples. A similar situation has also been seen but less distinctly in the hypocotyl patterns in the latter half of the germination stage (Figure 2). In contrast to this, the mobility of Component I', though it apparently rises daily, never draw near that of Component II' in the period examined. The same was true for Component I in the aged hypocotyls.

By the way, working with either the hypocotyls in the latter half of the germination stage or the germinating cotyledons, both being then largely catabolic in their metabolic activities (see the discussion), the yield of the centrifugal isolation of the microsome fraction was found to drop definitely day by day.

Table 1. *Extractability of microsomal phosphatid, RNA and protein with a phosphate buffer (pH 7.1, 0.1 M).* Lipid, RNA and protein fractions were isolated by a routine method of this laboratory (a combination of the Ogur-Rosen's and the Schneider's methods of fractionation, cf. Osawa and Oota). Total phosphorus of the phosphatid and the RNA fractions and total nitrogen of the protein fractions were determined by the King's and the Levy-Palmer's methods, respectively.

Tissues.....	1 day-old hypocotyls			1 day-old cotyledons		
	Microsome ($\mu\text{g}/250$ hypocotyls)	7.1-fraction ($\mu\text{g}/250$ hypocotyls)	Extract- ability (%)	Microsome ($\mu\text{g}/50$ pairs of cotyledons)	7.1-fraction ($\mu\text{g}/50$ pairs of cotyledons)	Extract- ability (%)
Phosphatid-P	416	140	34	176	50	28
RNA-P	835	722	87	584	549	94
Protein-N ...	2,170	1,890	87	1,970	1,630	83

Discussion

Our present impression is that Component II may be the essential ribonucleoprotein of the Palade granules or *functional RNA* in the hypocotyl microsomes. A convincing evidence will be given if labelled amino acid fed to the tissues will be shown to be predominantly incorporated into Component II. The slowest moving Component I may only be ascribed to fragmental particles of membranous structure, since the pH 7.1-fractions examined must be far from being free from the contamination of membranous fragments as indicated from their significant phosphatid contents (Table 1).

Our hypothesis insists that with the age or decayed protein producing activity of the tissues the nucleic acid moiety of *functional RNA* may be released from its protein partner. Thus at least in the aged hypocotyl tissues Components IV as well as III would be disintegration products of *functional RNA*, in other words, transitional steps from *functional RNA* to *transportable RNA*. The suggested sequence of conversion in the hypocotyl cytoplasm is described as: *functional RNA* (Component II) \rightarrow transient free RNA-s (III and IV) \rightarrow *transportable RNA*. In the early germination stage, however, it is probable that Component III is not a degradation product of Component II but a step of conversion in the reverse direction, i.e., from *transportable RNA* donated from the cotyledons to *functional RNA* (see the later comment on *transportable RNA* of the cotyledons).

The above assumption enables us to conclude that the increase in electrophoretic mobility or the increase in RNA concentration of a component is to be brought about not by the increase in RNA but by the decrease in protein of the component. Needless to say, the mobilities of free RNA-s should depend mainly on their size and/or electric charge. The protein thus released from nucleoproteins must be unextractable with the buffer solution used or concealed under such huge stationary boundary, since any detectable non-nucleoprotein peak is not contained in our electrophoretic diagrams.

A striking similarity found in the time course of change in electrophoretic pattern between the aged hypocotyls and the cotyledons is easily explainable. As has been repeatedly stressed, in the etiolation conditions studied the metabolic pattern of the hypocotyls of advanced ages is at least partly analogous to that of the germinating cotyledons. Thus with the termination of the first half of the germination stage the hypocotyls turn markedly catabolic in their metabolic activities and, in place of the already nearly exhausted cotyledons, begin to supply various nutrients to the actively growing portions, the epicotyls in particular where the most vigorous growth in the aged seedlings is carried out. The hypocotyls then deserve the name "secondary storehouse" for the germs which now can hardly rely on the cotyledons as "primary storehouse" for their demands for nutrients (Oota). Greater conspicuousness or regularity in the emergence of electrophoretic components of progressive mobilities in the cotyledons than in the aged hypocotyls (compare Figure 4 with Figure 2) will reflect more intense and simple catabolism prevailing in the former tissues.

The view of gradual disintegration of microsomal nucleoproteins with the age of tissues is supported by Lund *et al.* who have demonstrated electron-microscopically a ready diminution in population of microsomal (Palade) granules with the maturation of maize root cells. Decreased yield of microsome isolation with the age of tissues used in the present study also suggests progressive disintegration of microsomes in these aging tissues.

That the removal of the epicotyls at the mid-germination stage results in an accumulation of Component IV in the hypocotyl microsomes will be explained as well on the assumption that this (free) nucleic acid may be the antecedent of *transportable RNA*. In the normal plants, a very swift migration of *transportable RNA* from the hypocotyls up to the epicotyls would cause such a rapid removal of Component IV from the particulate particles of the former tissues that no detectable Component IV may be deposited there.

Assumingly Component II' of the cotyledon microsomes may be the remain of *functional RNA* which has probably participated in the active protein formation in the seed maturation stage (Oota and Osawa, unpublished, *cf.* Oota) and now is disintegrating to yield *transportable RNA* via Components III' and IV'. Thus formed *transportable RNA* will be transported to the growing seedling axes. The absence of the fastest moving component (IV') at the mid-germination stage is likely attributable to the loss exceeding the gain. Component I' may be membranous fragments of cotyledon microsomes as was assumed above for Component I of the hypocotyls.

Summary

Daily changes in microsomal ribonucleoproteins of the etiolated bean hypocotyls and cotyledons were investigated electrophoretically. The results obtained were considered along the line of the previously reported hypothesis which assumes interconversion of two existing forms of cytoplasmic ribonucleic acids, i.e., *functional* and *transportable RNA-s*.

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The Production of Indole Acetic Acid by *Taphrina deformans* and *Dibotryon morbosum*¹

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Introduction

Indole acetic acid is a metabolic product of a number of microorganisms, including certain fungi which parasitize higher plants and cause overgrowth of affected tissue, with hypertrophy or hyperplasia, or both. The present study is concerned with indole acetic acid production by *Taphrina deformans*, which parasitizes peach leaves, causing the disease known as peach leaf curl, and *Dibotryon morbosum*, which parasitizes plum and cherry trees, causing black knot disease.

Link, Wilcox and Link (1937) and Link, Wilcox and Eggers (1938) have briefly reported that culture filtrates of *Taphrina deformans* give a positive Salkowski test characteristic of indole acetic acid. No previous observations of this kind have been found for *Dibotryon morbosum*. Among the fungi known to produce indole acetic acid are *Rhizopus suinus* (Thimann 1935), *Nectria galligena* (Berduco 1949), *Ustilago zeae* (Moulton 1942, Wolf 1952), *Exobasidium camelliae* var. *gracilis* (Wolf and Wolf 1952) and a number of species of rusts (Pilet 1952, 1953, 1957, Hirata 1954, Wolf 1956, Daly and Inman 1958).

The biosynthesis of indole acetic acid from tryptophane may occur through either of two alternative pathways (Gordon and Sanchez Nieva 1949), in one

¹ From a thesis submitted by the senior author to the Graduate School of Vanderbilt University in partial fulfillment of the requirements for the Master of Science degree, June 1959.

of which indole pyruvic acid and indole acetaldehyde are the intermediates, while tryptamine and indole acetaldehyde are intermediates in the other. In most higher plants, synthesis proceeds by the indole pyruvic acid pathway. It was the purpose of this study to determine whether indole acetic acid is produced by *Dibotryon morbosum*, to determine the concentrations of indole acetic acid produced by both *T. deformans* and *D. morbosum* in stationary and shake cultures, and to attempt to determine the route of conversion of the precursor to indole acetic acid in these organisms.

Materials and Methods

Cultures of *Taphrina deformans* (Berk.) Tul., A.T.C.C. No. 11124, and *Dibotryon morbosum* (Schw.) T. and S., A.T.C.C. No. 11350, were obtained from the American Type Culture Collection, Washington, D. C. Stock cultures were grown on potato dextrose agar slants at room temperature.

Attempts were made to grow *T. deformans* in a synthetic medium according to Mix (1953). Because growth was inconsistent in this medium, potato dextrose broth was used as the basal medium for studies of indole acetic acid production by *T. deformans*. *Dibotryon morbosum* was grown in a synthetic medium containing dextrose 50.0 g., KH_2PO_4 1.5 g., $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5 g., FeCl_3 trace, and L-asparagine 2.0 g. per liter. To these basal media were added L-tryptophane 1.0 g./liter or tryptamine · HCl 1.0 g./liter. The pH of the synthetic medium for *Dibotryon* was adjusted to approximately 5.5 by the addition of NaOH; pH was not adjusted in the potato dextrose broth.

All media were distributed in 50 ml. portions in 125 ml. Erlenmeyer flasks, which were sterilized by autoclaving for 15 minutes at 15 pounds pressure. Inoculations were made from spore suspensions in sterile distilled water, prepared from cultures grown on slants of potato dextrose agar. Uninoculated flasks served as controls. The inoculated flasks were incubated on a rotary shaker operated at a speed of 140 r.p.m., or under stationary conditions at room temperature.

At intervals during the incubation period, colorimetric tests for the presence of indole acetic acid were made on culture filtrates, using the ferric chloride-perchloric acid reagent and procedure of Gordon and Weber (1951). To a 2-ml. aliquot of the culture filtrate, 4 ml. of the reagent were added, and, after standing 25 minutes for color development, optical density readings at 530 m μ were taken with a Beckman model DU spectrophotometer. Uninoculated medium, to which the reagent was added, served as a blank in all spectrophotometric determinations.

In order to convert optical density readings into units of indole acetic acid concentration, dilutions of an authentic solution of indole acetic acid were prepared ranging from 5 to 100 $\mu\text{g/ml.}$, treated with the ferric chloride-perchloric acid reagent, and their optical densities were measured as before. The results indicated a linear relation between indole acetic acid concentration and optical density within this range of concentrations.

Since the colorimetric method is not specific for indole acetic acid, unidimensional ascending paper chromatography was employed, according to Sen and Leopold (1954) and Wolf (1956). All cultures used in chromatographic work were incubated

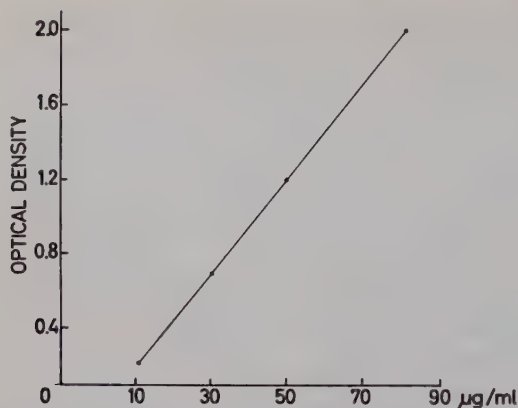


Figure 1. The relation between indole acetic acid concentration and optical density.

on the shaker, since it was found that higher yields of indole acetic acid were produced in less time than in stationary cultures. Culture filtrates were acidified with HCl, extracted twice with ether in a separatory funnel, and the ether extracts were then evaporated almost to dryness.

Strips of Whatman number 1 filter paper were spotted with the extract, and developed with either isopropanol : ammonia : water, 10 : 1 : 1 by volume, or with 70 per cent ethanol. After development, the paper strips were sprayed with the nitrite reagent of Mitchell and Brunstetter (1939).

Results

When known concentrations of indole acetic acid were allowed to react with the ferric chloride-perchloric acid reagent, and the optical density of the solution at 530 m μ was determined with the spectrophotometer, a linear relationship between optical density and indole acetic acid concentration was found (Figure 1). This standard curve was employed in translating optical density measurements made on culture filtrates into concentrations of indole acetic acid.

Cultures of *Taphrina deformans* grown on a shaker and under stationary conditions in the basal medium supplemented with 1.0 gm. of L-tryptophane per liter were assayed for indole acetic acid at daily intervals during the incubation period of 6—7 days. When it was found that shake cultures of *T. deformans* formed quite high amounts of indole acetic acid within 24 hours, the interval between successive tests was decreased to 4—6 hours for the first day of incubation. The results of two typical experiments with stationary cultures are presented in Figure 2. No indole acetic acid was detected within 1 day, but after 2 days the level had risen to 9.2 μ g./ml. The increase was practically linear between 2 and 6 days, the highest level reached

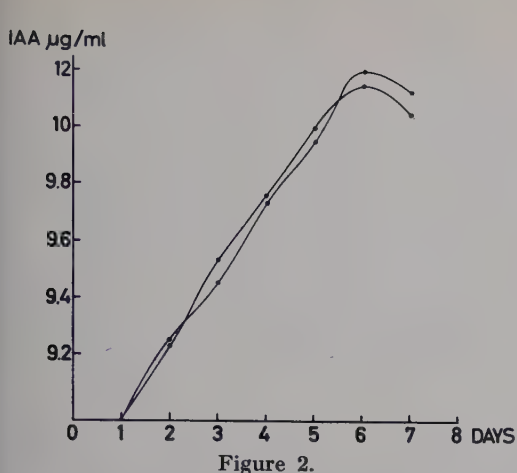


Figure 2.

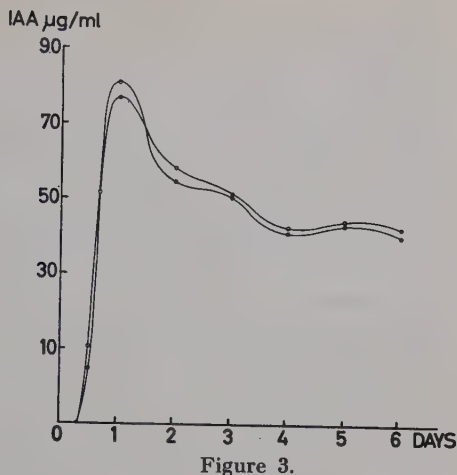


Figure 3.

Figure 2. Indole acetic acid concentration as a function of time in stationary cultures of *Taphrina deformans*.

Figure 3. Indole acetic acid concentration as a function of time in shake cultures of *Taphrina deformans*.

being 12.0 µg./ml. at 6 days time. In shake cultures (Figure 3) synthesis of indole acetic acid is considerably more rapid than in stationary cultures. The highest yields are reached after only one day of incubation and gradually decline thereafter. The highest concentration of indole acetic acid attained in shake cultures was 81.2 µg./ml., essentially 7 times as great as that obtained in stationary cultures. When one considers that highest yield in stationary cultures was obtained only after 6 days incubation, it is apparent that biosynthesis of indole acetic acid proceeds some 40 times faster in shake cultures than in stationary cultures of *T. deformans* grown on the same medium.

The results of similar experiments with *Dibotryon morbosum*, which grows much more slowly than *Taphrina*, are presented in Figures 4 and 5. In stationary cultures (Figure 4) no indole acetic acid was detected after 6 1/2 days of incubation. Measurable quantities were first found after 11 days, and the titer gradually increased with time until 20 days or more. The highest concentration found was 12.1 µg./ml. at 20 days of incubation. In shake cultures (Figure 5) detectable quantities of indole acetic acid became apparent after 6 1/2 days of incubation, and the concentration increased almost linearly until 15—17 days. The highest titer attained was 20.5 µg./ml., again, as in the case of *Taphrina*, higher than in stationary cultures.

Following these experiments in which some information was gained as to the levels of indole acetic acid produced, and the time required for maximal yield, culture filtrates of shaker grown cultures of both *T. deformans* and

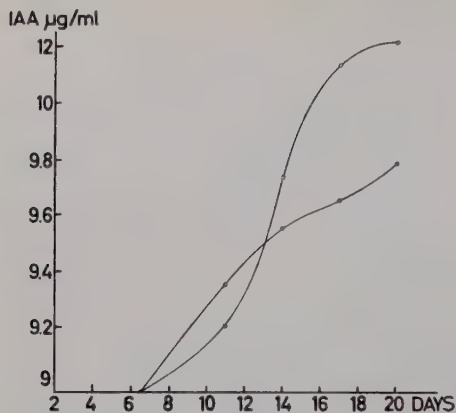


Figure 4.

Figure 4. Indole acetic acid concentration as a function of time in stationary cultures of *Dibotryon morbosum*.

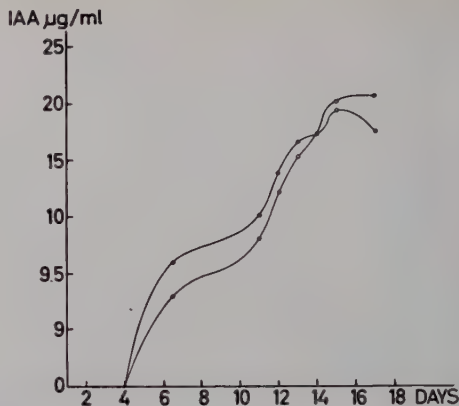


Figure 5.

Figure 5. Indole acetic acid concentration as a function of time in shake cultures of *Dibotryon morbosum*.

D. morbosum were acidified, extracted with ether, and the concentrated ether extracts were chromatographed in order to ascertain that the substance responsible for the colorimetric tests was actually indole acetic acid. Since this proved to be the case, efforts were then made to determine the nature of the intermediate in the transformation of tryptophane.

Upon chromatography of culture filtrates of *T. deformans*, grown on a medium containing tryptophane, two spots were routinely found after development and spraying. With isopropanol : ammonia : water, 10 : 1 : 1 by volume, as the developing solvent mixture, there was a red spot with an R_F of 0.30 and a purple spot with an R_F of 0.73. When 70 per cent ethanol was used for development, the purple spot was found at an R_F of 0.76 and the red spot at an R_F of 0.88. By reference to the data of Sen and Leopold (1954) as well as chromatography with authentic known compounds, it was established that the red spot corresponds to indole acetic acid and the purple spot to tryptamine.

Essentially similar findings were obtained with culture filtrates of *D. morbosum* grown upon a medium containing tryptophane. With isopropanol : ammonia : water, 10 : 1 : 1 by volume, as the developing solvent, there were found a yellow spot with an R_F of 0.14, a red spot with an R_F of 0.48, and a purple spot with an R_F of 0.77. These were identified as tryptophane, indole acetic acid, and tryptamine, respectively. On chromatography with 70 per

cent ethanol, a purple spot was found with an R_F of 0.81 and a red spot with an R_F of 0.91, identified as tryptamine and indole acetic acid, respectively.

Following the experiments in which tryptamine was shown to be present in culture filtrates of both *T. deformans* and *D. morbosum*, these organisms were grown in the basal media supplemented with 1.0 gm. per liter of tryptamine·HCl. After incubation, extraction of the culture filtrate, and chromatography, a red spot was found on the chromatogram of the culture filtrate of *T. deformans* (R_F in 70 per ethanol, 0.85) which was identified as indole acetic acid. Similarly, with *D. morbosum*, a red spot was found (R_F in isopropanol : ammonia : water, 10 : 1 : 1, 0.44) which was identified as indole acetic acid.

Discussion

The present data indicate that indole acetic acid is produced by both *T. deformans* and *D. morbosum*. The highest concentrations found were 20.5 $\mu\text{g./ml.}$ for Dibotryon and 81.2 $\mu\text{g./ml.}$ for Taphrina. Bearing in mind that maximum yield of indole acetic acid in shake cultures of *T. deformans* was reached at 1 day, while that of *D. morbosum* was attained only at 15—17 days of incubation, it is clear that the rate of indole acetic acid synthesis is some 60 times faster for Taphrina than for Dibotryon. In partial explanation of this finding, it may be noted that *T. deformans* grows in culture in a yeast-like fashion, and its growth rate is very high, while *D. morbosum*, a filamentous fungus, grows far more slowly. Similarly, the proliferation of the host plant induced by *T. deformans* may occur within a very short period, possibly two weeks, following inoculation, while in *D. morbosum* infections the symptoms are much slower to appear, and two years are required for maximum development.

From the present experiments, it may be calculated that 9.5 per cent of the tryptophane originally present in the media is converted to indole acetic acid by *T. deformans*, and 2.4 per cent conversion occurs by *D. morbosum*.

In almost all higher plants, indole pyruvic acid is a normal intermediate in the conversion of tryptophane to indole acetic acid. Relatively few instances are known, such as in pineapple leaves (Gordon and Sanchez-Nieva 1949) in which indole acetic acid may arise from tryptamine. The identification of a spot corresponding to tryptamine on paper chromatograms of filtrates of cultures of both *T. deformans* and *D. morbosum* grown on media containing tryptophane, and the identification of a spot corresponding to indole acetic acid on paper chromatograms of filtrates containing tryptamine strongly supports the idea that tryptamine is the normal intermediate in indole acetic acid biosynthesis in both of these fungi. These findings are essentially similar

to observations reported earlier on the rust *Gymnosporangium juniperi-virginianae* (Wolf 1956).

The problem of relating overgrowths in parasitized plant tissues to indole acetic acid produced by the parasite still remains obscure. Brian (1957) has pointed out some of the difficulties in considering overgrowth in a parasitized tissue as a result of indole acetic acid production by the parasite. Recent studies by Pilet (1957) with *Uromyces pisi* and Shaw and Hawkins (1958) with wheat tissues parasitized by *Puccinia graminis tritici* or *Erysiphe graminis* have served to focus attention on the activity of indole acetic acid oxidase in host and parasite rather than on the synthesis of indole acetic acid. Still further investigations are needed in order to bring a definitive answer to this problem.

Summary

Taphrina deformans and *Dibotryon morbosum*, when grown in culture in media containing tryptophane, produce indole acetic acid. The highest concentrations of indole acetic acid found by colorimetric tests were 20.5 µg./ml. in the case of *D. morbosum* and 81.2 µg./ml. in the case of *T. deformans*. The rate of biosynthesis, as well as the total quantity of indole acetic acid produced, is greater for *T. deformans* than for *D. morbosum* and is greater in shake cultures than in stationary cultures. Evidence obtained by paper chromatography strongly favors tryptamine as the probable intermediate in the conversion of tryptophane to indole acetic acid by both of these fungi.

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Die Permeabilität der Hefezellen für Säuren

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Einleitung

Über die Permeabilität der Hefezellen für Säuren liegen bisher nur ziemlich summarische Angaben vor. So z.B. fand Malm (1950), dass Ameisen-, Essig-, Propion-, Butter-, Isobutter- und β -Oxybuttersäure in weniger als einer Minute bis zum Konzentrationsausgleich in die Zellen der Bäckerhefe eindringen, wogegen Bernstein-, Wein- und Citronensäure in 20 Minuten nicht merkbar aufgenommen werden. Gleichzeitig stellten Conway und Downey (1950) fest, dass Oxal-, Milch-, Brenztrauben-, Glycerin-, Bernstein-, Fumar-, Äpfel-, Wein-, Citronen- und Gluconsäure in einer Stunde nicht merkbar eindringen. „After 24 hr. there appears to have been a marked entrance of lactic acid, and of fumaric acid to a somewhat lesser degree. Appreciable entrance of malic also appears to have occurred after 24 hr.“ Dagegen permeierten die geprüften Fettsäuren, d.h. Ameisen-, Essig-, Propion- und Buttersäure „almost at once“. Ebenso fanden zwei von uns (Suomalainen und Oura 1955) sowohl mit Bäcker- als mit Brauereihefe, dass Fettsäuren schnell in Hefezellen eindringen, wogegen Citronen- und Weinsäure praktisch gar nicht und Bernsteinsäure nur sehr langsam permeierten, während Milchsäure eine Zwischenstellung einnahm. Der seit langem bekannte Unterschied zwischen Brauerei- und Bäckerhefe hinsichtlich ihres Verhaltens alkaligepufferten Lösungen gegenüber hat die Vermutung veranlasst, dass die Brauereihefe durchlässiger als die Bäckerhefe sei. Bezüglich der Säurepermeabilität ist diese Vermutung bestätigt worden (Suomalainen und Oura 1955, 1958 a). Sowohl mit Bäcker- wie mit Brauereihefe wurde ausserdem

festgestellt, dass das Permeationsvermögen der α -Ketosäuren von der Brenztraubensäure bis zur Ketokaprylsäure mit wachsender Länge der Kohlenstoffkette ziemlich gleichmässig zunimmt (Suomalainen und Oura 1957, 1958 a, b).

Es lag nun nahe, diese Versuche in quantitativer Hinsicht etwas weiter auszubauen. Wie bei den Versuchen von Malm sowie bei denjenigen von Conway und Downey sind wir von der Annahme ausgegangen, dass sich das Eindringen einer zu der Hefesuspension zugesetzten Säure in die Zellen als eine entsprechende Erhöhung des pH-Wertes der Aussenlösung bemerkbar machen müsse. Derartige Versuche sind von Interesse u.a. in der Hinsicht, dass die Permeation der Säuren dabei — jedenfalls in erster Linie — von der Durchlässigkeit des Plasmalemmas abhängt, über dessen Permeabilitätseigenschaften sehr divergierende Meinungen geäussert worden sind (vgl. Collander 1956).

Über die von uns erzielten Ergebnisse ist eine vorläufige Mitteilung bereits erschienen (Oura, Suomalainen und Collander 1958).

Versuchsmethodik

Wir nehmen an, dass zu einer Hefesuspension eine Säure hinzugegeben wird, deren Gesamtkonzentration in der Aussenlösung unmittelbar nach dem Zusatz, d.h. zur Zeit t_0 , C_0 beträgt. Der entsprechende pH-Wert der Aussenlösung sei p_0 . Da die Säure unmittelbar anfängt, in die Zellen einzudringen, lässt sich p_0 im allgemeinen nicht direkt, sondern nur durch Extrapolation bestimmen. In dem Masse, wie die Säure in die Zellen eindringt, steigt der pH-Wert der Aussenlösung und erreicht zur Zeit t den Wert p . Die Konzentration der in der Aussenlösung zurückgebliebenen Säure beträgt dann C . Wenn das Volumen der Aussenlösung V beträgt, so ist die Differenz $C_0V - CV$ ein Mass der aus der Aussenlösung in die Zellen übergetretenen Säuremenge. Diese Säuremenge ist der Basenmenge äquivalent, die erforderlich ist, um die gleiche Erhöhung des pH-Wertes in einer entsprechenden Menge der Aussenlösung zu bewirken.

Um die Permeationskonstanten der Säuren zu berechnen, gehen wir von der folgenden Gleichung (vgl. Åyräpää 1950, Gleichung 1,1) aus:

$$\frac{dc}{dt} = \frac{Pq}{v} (pC - \beta c) \quad (1)$$

Hierin bedeutet c die intrazelluläre Gesamtkonzentration der Säure zur Zeit t , P die Permeationskonstante, q die Gesamtoberfläche der suspendierten Hefezellen, v das Volumen der Hefezellen, während q und β den Dissoziationsrest ($\frac{HA}{HA + A^-}$) der Säure in der Aussenlösung bzw. im Zellinnern bezeichnen. Die Bedeutung der übrigen Symbole ist oben bereits angegeben.

Um die Integrierung zu ermöglichen, nehmen wir an, dass die geprüften Säuren

praktisch vollständig im Protoplasma der Hefezellen, dessen pH-Wert etwa bei 5,8—6,0 liegen dürfte¹, dissoziiert sind, so dass β also praktisch gleich 0 ist. Dagegen lässt sich q in den meisten Versuchen gleich 1 setzen. C kann leicht als eine Funktion von c ausgedrückt werden: $C = C_0 - \frac{v}{V}c$. Wenn dieser Wert von C in die Gleichung (1) eingesetzt wird, ergibt sich durch Integration:

$$P = \frac{V}{q\Delta t} \ln \frac{C_0}{C_0 - \frac{v}{V}c} \quad (2)$$

Da $\frac{v}{V}c \leq \frac{1}{4} C$, ergeben sich (vgl. Åyräpää 1950, S. 412) die weiteren Gleichungen

$$P = \frac{V \frac{v}{V} \cdot c}{q\Delta t \frac{C_0 + (C_0 - \frac{v}{V}c)}{2}} \quad (3)$$

und

$$P = \frac{2 Vvc}{q\Delta t (2 C_0 V - vc)} \quad (4)$$

Da wir Grund haben zu der Annahme, dass praktisch allein die undissoziierten Säuremoleküle (HA) permeationsfähig sind, sind die in Klammern stehenden Grössen C_0 und c durch C_{HA} und c_{HA} zu ersetzen. Die Grösse $c_{HA}v$ ist sehr klein verglichen mit $2 C_{HA}V$ und kann daher im Divisor vernachlässigt werden. Die Gleichung (4) nimmt somit die folgende Form an:

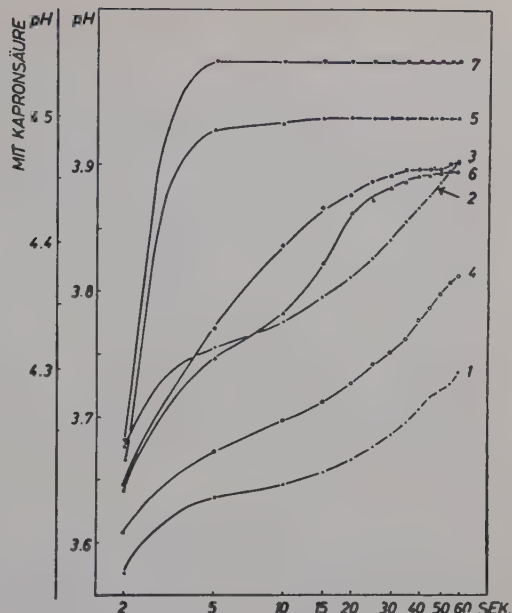
$$P = \frac{vc}{q\Delta t C_{HA}} \quad (5)$$

Dies ist die Gleichung, die bei der Berechnung der P-Werte benutzt wurde. Für q ist der Wert 3,88 m²/g Frischgewicht Hefe benutzt worden, den Just (1940 a) für Brauereihefe bestimmt hat.

Zu den Versuchen wurden Hefeproben verschiedener Herkunft verwendet. Die Bäckerhefe stammte aus den Rajamäki-Werken des Staatlichen Alkoholmonopols. Die meisten Versuche wurden mit der streng aerob gezüchteten käuflichen Bäckerhefe-stufe A₅ durchgeführt. Vergleichsweise wurden auch die Permeabilitätseigenschaften der mehr anaerob gezüchteten Vorstufen A₃ und A₄ geprüft. Die Brauereihefe stammte aus der Brauerei Oy Mallasjuoma, Lahti. Die Hefeproben wurden unmittelbar vor dem Gebrauch mit Leitungswasser gewaschen, wonach das Waschwasser im Büchner-Trichter abgesaugt wurde, so dass Hefekuchen von etwa 25 % Trocken-gewichtsgehalt erhalten wurden. Von der Bäckerhefe wurden Suspensionen 1 : 1

¹ Die intracelluläre Azidität der Hefe wurde in der Weise untersucht, dass die Hefe in einem Kohlensäureschnee-Äthanol-Gemisch gefroren und in lauwarmen Wasser aufgetaut wurde, wonach der pH-Wert des Desintegrats unmittelbar bei Zimmertemperatur bestimmt wurde. Ergebnis mit Brauereihefe pH 5,98, mit käuflicher Bäckerhefe pH 5,79 (vgl. z.B. Caldwell 1956). Der pH-Wert des Protoplasmas dürfte aber etwas höher liegen als derjenige des Desintegrats, das ja auch den Zellsaft enthält.

Abbildung 1. Zeitliche Veränderung des pH-Wertes der Suspensionsflüssigkeit nach Zugabe von Fettsäuren als 0,3-N Lösungen (die Kapronsäure jedoch als 0,04-N) zu einer Suspension von käuflicher Bäckerhefe. Abszisse: Zeit in Sekunden, gerechnet von der Zugabe der Säurelösung, Masstab logarithmisch. Essigsäure (1), Propionsäure (2), Buttersäure (3), Isobuttersäure (4), Valeriansäure (5), Isovaleriansäure (6) und Kapronsäure (7).



(Gew./Vol.) in Leitungswasser oder Pufferlösung hergestellt. Die Brauereihefe wurde dagegen im Verhältnis 1 : 2 suspendiert. Das pH der Hefesuspension (ohne Säurezusatz) betrug etwa 4,5—5,2 im Falle der Bäckerhefe und etwa 5,2—5,4 im Falle der Brauereihefe¹. Da diese pH-Werte wenigstens 20—30 Minuten praktisch unverändert blieben, waren besondere Blindversuche ohne Säurezusatz nicht erforderlich.

Reinste im Handel erhältliche Säurepräparate wurden benutzt, meistens als 0,3-N Lösungen. Nur die Brenztraubensäure wurde vor dem Gebrauch im Vacuum destilliert. Sowohl die Säurelösungen wie die Hefesuspensionen wurden vor dem Versuchsbeginn auf 22°C. temperiert.

Zu den pH-Messungen wurde ein Potentiometer der Radiometer-A.G., Kopenhagen (Modell PHM 3f, mit Glas- und Kalomelelektroden) herangezogen. Durch Zusammenarbeit von zwei Personen war es möglich auch schnelle Veränderungen des pH-Wertes zu verfolgen.

Der Versuchsgang war folgender: 50 ml Hefesuspension wurde in ein Becherglas gebracht und die Elektroden des pH-Meters sowie der Rührer in die Suspension eingesenkt, wonach der Rührer in Gang gesetzt wurde. Nun wurden 5 ml Säurelösung rasch mittels einer Injektionsspritze der Suspension zugefügt und die Ausschläge des Instrumentes verfolgt. Der erste pH-Wert wurde nach 2 Sekunden notiert, die folgenden nach 5, 10, 15 u.s.w. bis 60 Sek. vom Versuchsbeginn an gerechnet. Alle Versuche wurden in duplize ausgeführt. Nach dem die zeitliche Veränderung des pH-Wertes in der Suspension festgestellt worden war, wurde eine Hefesuspension ganz

¹ Merkwürdigerweise wurden um 0,1—1,0 Einheiten höhere pH-Werte in der abgesaugten Suspensionsflüssigkeit gefunden als wenn die Messung direkt in der Suspension vorgenommen wurde. Dieser Unterschied kann nicht allein auf das Entweichen von Kohlensäure beim Filtrieren zurückgeführt werden.

derselben Zusammensetzung aber ohne Säurezusatz filtriert, wonach dem Filtrat eine Menge entnommen wurde, die dem Volumen der extrazellulären Flüssigkeit entsprach. Hierbei wurde angenommen, dass das extrazelluläre Wasser des 25 % Trokensubstanz enthaltenden Hefekuchens 26 Volumprozent betrug (White 1954, Conway und Downey 1950, Just 1940 b). Zu der betreffenden Filtratmenge wurden 5 ml der im Versuch benutzten Säurelösung hinzugefügt und darauf mittels 0,1-N NaOH-Lösung die Titrationskurve der Lösung ermittelt. Aus einer solchen Kurve ist direkt ersichtlich, wie viel Base erforderlich ist, um im Filtrat dieselbe pH-Steigerung zu bewirken, wie sie beim Versuch in der Hefesuspension beobachtet wurde. Die während des Versuchs in die Hefezellen eingedrungene Säuremenge wurde als dieser Basenmenge äquivalent betrachtet.

Mehrere Rührer verschiedener Art wurden auf ihre Effektivität hin geprüft. Ein durchlöcherter birnförmiger Rührer erwies sich dabei als am zweckmässigsten. Durch hinzufügen von Farblösung wurde visuell festgestellt, dass vollständige Durchmischung in 1—2 Sek. erreicht wurde.

Beim Berechnen der P-Werte wurde allein das Zeitintervall von 2—5 Sek. berücksichtigt.

Versuche mit Fettsäuren

Versuche über das Eindringen einiger Fettsäuren aus ungepufferten Lösungen in käufliche Bäckerhefe ergaben die in Abb. 1 dargestellten Resultate. Tabellen 1 und 2 geben wiederum die auf Grund ähnlicher Versuche berechneten Werte der Permeationskonstanten derselben Säuren verschiedenen Hefetypen gegenüber.

Die erhaltenen Versuchsergebnisse können leider auf keinen hohen Grad der Genauigkeit Anspruch erheben. Das ergibt sich erstens daraus, dass die Permeationszeit, die der Berechnung der P-Werte zugrundegelegt wurde, nur 3 Sek. betrug. Infolgedessen können bereits kleine Fehler hinsichtlich der Zeitbegrenzung die Ergebnisse stark beeinflusst haben. Ausserdem waren

Tabelle 1. *Permeationskonstanten einiger Fettsäuren in Bezug auf unter kräftiger Belüftung gezüchtete käufliche Bäckerhefe (A₅-Stufe), ihre weniger aerob gezüchteten Vorstufen (A₃- und A₄-Stufe) und anaerob gewachsene Brauereihefe.*

Säure	Bäckerhefe 10 ⁻⁸ cm sek ⁻¹						Brauereihefe 10 ⁻⁸ cm sek ⁻¹	
	A ₃ -Stufe	A ₄ -Stufe	A ₄ -Stufe	A ₄ -Stufe	A ₅ -Stufe	A ₅ -Stufe		
Essigsäure	13.0	14.5	10.7	10.7	22.6	18.8	58.9	56.8
Propionsäure	26.7	18.5	16.3	16.2	43.5	26.4	116.0	94.8
n-Buttersäure	25.5	23.9	25.6	25.7	43.8	43.5	174.7	133.8
Isobuttersäure	19.8	25.6	16.6	16.6	24.5	19.6	111.0	60.7
n-Valeriansäure . . .	63.8	66.4	41.3	69.7	105.7	116.0	274.8	199.4
Isovaleriansäure . . .	23.4	22.3	16.2	18.8	41.2	27.3	147.0	79.8
n-Kaprönsäure	275.5	197.5	117.3	91.7	387.0	447.0	804	674

24—48 h gelagerte Hefeproben.

Tabelle 2. *Permeationskonstanten einiger Fettsäuren in Bezug auf die als Anstellhefe dienende A₄-Stufe, während dem Wachstum der käuflichen A₅-Stufe sowie der handelsüblichen Bäckerhefe.*

Säure	A ₄ -Stufe ¹ 10 ⁻⁸ cm sek ⁻¹	A ₅ -Stufe ² 10 ⁻⁸ cm sek ⁻¹			
		6 h	12 h	16 h	fertig, 18 h
Essigsäure	10.7	(6.9)	18.1	19.1	23.9
Propionsäure	16.2	23.8	42.8	44.2	45.7
n-Buttersäure	25.7	42.3	108.5	135.6	92.0
Isobuttersäure	16.6	120.1	—	72.3	48.7
n-Valeriansäure	69.7	93.5	194	159.5	126.0
Isovaleriansäure	18.8	24.1	67.6	113.4	68.4
n-Kaprönsäure	91.7	526	—	514	—

¹ 24—48 h gelagerte Hefeproben.

² Unter 12 h gelagerte Hefeproben.

auch die während der Versuchszeit auftretenden Veränderungen des pH-Wertes sehr klein. Absolut genommen kleine Fehler bei den pH-Bestimmungen beeinflussten somit die berechneten Werte der Permeationskonstanten stark.

Einige Versuche wurden auch mit Hefesuspensionen ausgeführt, die mittels Citrat-Phosphat-Gemischen schwach gepuffert worden waren. Die betreffenden Suspensionen enthielten 50 Proz. (Gew./Vol.) käufliche Bäckerhefe. Zu je 50 ml der gepufferten Suspension wurden 5 ml eines 0,3-molaren Natriumacetat-Essigsäure-, bzw. eines Natriumvalerat-Valeriansäure-Gemisches hinzugefügt, dessen pH-Wert mit demjenigen der betreffenden Suspension übereinstimmte. Die Permeationskonstanten wurden in diesen Versuchen auf Grund des Anstiegens des pH-Wertes während des Zeitintervalls 0—3 Sek. berechnet, wobei als pH-Wert zur Zeit 0 der ursprüngliche pH-Wert der betreffenden Suspension angenommen wurde. Wie aus Tabelle 3 ersichtlich, wurden in dieser Weise Werte der Permeationskonstanten erhalten, die mit ansteigendem pH-Wert der Suspension beträchtlich zunahmen. Bei der durchgeführten Probe mit ungepuffelter Suspension und Zusatz von 0,3-N Säurelösung wurden bei der angewendeten Bäckerhefe $P_{\text{Essigs.}} 11,2 \times 10^{-8}$ und $P_{\text{Valerians.}} 96,5 \times 10^{-8}$ cm × sek⁻¹ erhalten.

Folgende Resultate dürften trotz der grossen Variationsamplitude als gesichert angesehen werden können:

1. Fettsäuren mit unverzweigten Kohlenstoffketten permeierten um so schneller, je länger ihre Kohlenstoffkette und je grösser somit auch ihre relative Lipidlöslichkeit ist. In der Tat sind die Permeationskonstanten dieser Säuren, wie aus Abb. 2 ersichtlich, in erster Annäherung ihrer relativen Ätherlöslichkeit direkt proportional. Doch scheint zugleich auch die Molekülgrösse — direkt oder indirekt — hierbei eine Rolle zu spielen, in dem die kleinsten Säuremoleküle etwas schneller permeieren als allein auf Grund ihrer Ätherlöslichkeit zu erwarten wäre. Und zwar scheinen die permeations-

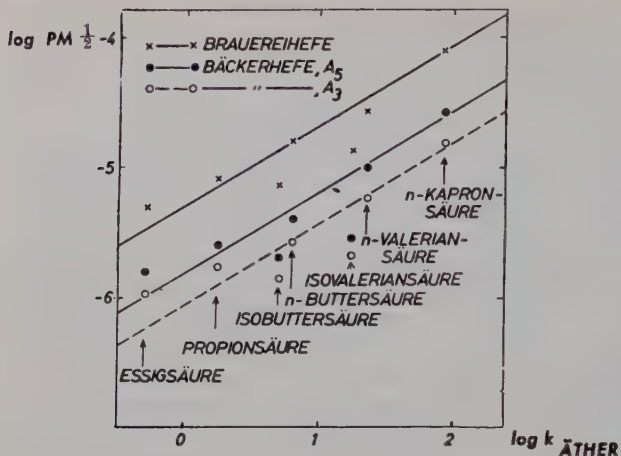


Abbildung 2. Permeationskonstanten einiger Fettsäuren gegenüber den Zellen der Brauereihefe sowie gegenüber aerob (A_5) und halb-aerob (A_3) gewachsenen Zellen der Bäckerhefe bezogen auf die relative Ätherlöslichkeit dieser Säuren (Ätherlöslichkeit nach Collander 1949).

konstanten nicht nur der Quadratwurzel des Molekulargewichts sondern einer etwas höheren Potenz desselben umgekehrt proportional zu sein. Um eine wie hohe Potenz es sich dabei handelt, lässt sich jedoch auf Grund unserer Versuche kaum entscheiden.

2. Aus Abb. 2 ist ferner ersichtlich, dass die beiden untersuchten verzweigt-kettigen Säuren, die Isobutter- und Isovaleriansäure, bedeutend langsamer permeierten als auf Grund ihrer Ätherlöslichkeit und Molekülgrösse zu erwarten wäre. Unerwartet war dieser Befund allerdings nicht, da es sich auch in einigen anderen Fällen gezeigt hat, dass sperrig gebaute Moleküle *ceteris paribus* wesentlich langsamer permeieren als geradkettige (Suomalainen und Oura 1957, 1958 a, Collander 1959, Wartiovaara und Collander 1959).

3. Versuche mit verschiedenen Bäckerhefestufen zeigten, wie aus Tabellen 1—2 und Abb. 2 ersichtlich, dass die Durchlässigkeit der aerob gezüchteten käuflichen Bäckerhefe etwa zweimal grösser als die der mehr anaerob gewachsenen Vorstufen A_3 und A_4 ist (vgl. auch Suomalainen und Oura 1958 a, b, 1959). Während der Züchtung der käuflichen Bäckerhefe nimmt die Permeabilität anfangs langsam zu und ist noch im Anfang der logarithmischen Wachstumsphase etwa von derselben Grösse wie bei der im Ruhezustand befindlichen Ausgangshefe (Tabelle 2). Beim Übergang zur stationären Phase wird aber die Durchlässigkeit verdoppelt und erreicht dabei dieselbe Grösse wie bei der reifen Handelshefe (vgl. auch Suomalainen und Oura 1959). Inwieweit aber Unterschiede hinsichtlich Zellgrösse oder Zellform für die gefundenen Permeabilitätsdifferenzen verantwortlich sind, ist nicht näher untersucht worden. Immerhin ist zu bemerken, dass die Zellgrösse der Bäckerhefe sogar in den anaeroben Stufen kleiner ist als diejenige der Brauereihefe und dass sie bei Verstärkung der Belüftung von Stufe zu Stufe noch kleiner wird.

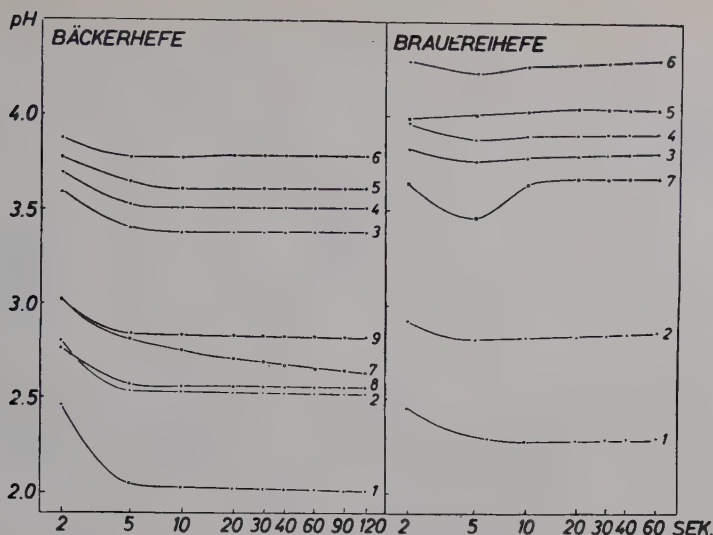


Abbildung 3. Zeitliche Veränderung des pH-Wertes der Suspensionsflüssigkeit nach Zugabe von zwei- und dreibasischen Säuren als 0,3-N Lösungen (die Adipin- und α -Ketoglutarsäure als 0,15-N) zu einer Suspension von Bäcker- oder Brauereihefe. Abszisse: Zeit in Sekunden, gerechnet von der Zugabe der Säurelösung, Masstab logarithmisch. Oxalsäure (1), Malonsäure (2), Bernsteinsäure (3), Glutarsäure (4), Pimelinsäure (5), Adipinsäure (6), α -Ketoglutarsäure (7), Weinsäure (8) und Citronensäure (9).

Versuche mit anderen Säuren

Ausser mit Fettsäuren wurden Versuche auch mit Oxyfettsäuren (Glykol-, Milch-, β -Oxybutter- und α -Oxyisobuttersäure), mit Dicarbonsäuren (Oxal-, Malon-, Bernstein-, Glutar-, Adipin- und Pimelinsäure) sowie mit Wein-, Citronen- und α -Ketoglutarsäure in der bereits beschriebenen Weise ausgeführt. Ein deutlicher Anstieg des extracellularen pH-Wertes konnte aber in Anwesenheit dieser Säuren nicht beobachtet werden. Eine Permeation dieser Säuren innerhalb der von uns gewählten Zeitspanne war also nicht festzustellen. Dies gilt auch für die β -Oxybuttersäure, für die Malm (1950) ein sehr schnelles Eindringen angibt.

Nach der Zugabe der obenerwähnten Säuren wurde zunächst eine Erniedrigung des extrazellularen pH-Wertes beobachtet, die erst nach rund 10 Sek. beendet war (Abb. 3). Eine plausible Erklärung dafür, dass das Absinken des pH-Wertes auf seinen definitiven Wert eine so lange Zeit in Anspruch nimmt, können wir nicht geben. Mangelnde Effektivität der Rührung oder eine träge Einstellung des pH-Meters scheinen hierbei nicht die entscheidenden Faktoren zu sein.

Auch mit α -Ketofettsäuren (Brenztrauben-, α -Ketobutter-, α -Ketovalerian-

Tabelle 3. Die mit Essig- und Valeriansäure erhaltenen Werte der Permeationskonstanten bei auf verschieden Säuregrade gepufferten Suspensionen käuflicher Bäckerhefe.

pH der Suspension und des zugesetzten Puffers	$P_{\text{Essigs.}} \times 10^{-8} \text{ cm sek}^{-1}$	$P_{\text{Valerians.}} \times 10^{-8} \text{ cm sek}^{-1}$
3.8	15.1	116.9
4.0	15.3	113.9
4.6	24.6	(96.0)
5.0	32.6	129.0
5.8	35.6	155.2
6.2	—	165.5
6.7	—	177.9

sowie α -Ketoisovaleriansäure) wurden Kurven erhalten, die durch ein Absinken des pH-Wertes der extrazellulären Flüssigkeit während der ersten 20—30 Sekunden charakterisiert waren, so dass auch sie sich nicht für die Berechnung von Permeationskonstanten eignen. Doch scheint das Permeationsvermögen auch innerhalb dieser homologen Reihe mit zunehmender Länge der Kohlenstoffkette zu wachsen, während die einzige geprüfte Iso-säure, die α -Ketoisovaleriansäure, bedeutend langsamer als die entsprechende normale Säure einzudringen scheint (vgl. Suomalainen und Oura 1957, 1958 a).

Vergleich mit früheren Befunden

Die vorangehend dargestellten Ergebnisse stimmen gut mit den mehr qualitativen Resultaten von Malm (1950) sowie von Conway und Downey (1950) überein. Nur in bezug auf die β -Oxybuttersäure besteht, wie bereits bemerkt, eine deutliche Diskrepanz zwischen den Angaben Malms und unseren Befunden.

Auch mit den Angaben eines von uns (Collander 1957) über die Durchlässigkeit des Plasmalemmas der Blattzellen von Elodea stimmen die hier geschilderten Befunde an Hefezellen in grossen Zügen überein. Ein quantitativer Vergleich wird allerdings u.a. dadurch erschwert, dass im Falle der Elodea-Zellen die verhältnismässig langsam permeierenden Säuren die klarsten Resultate ergaben, wogegen in der vorliegenden Untersuchung nur die schnell permeierenden Fettsäuren einigermaßen genau untersucht wurden. Auffallend ist unter allen Umständen, dass die Adipin- und besonders die Pimelinsäure nur wenig langsamer als Essig- und Propionsäure in die Elodea-Zellen einzudringen scheinen, wogegen der Unterschied zwischen diesen Säuren hinsichtlich ihres Eindringens in die Hefezellen sehr gross erscheint. Die Ursache dieser Diskrepanz können wir nicht angeben.

Dass das Permeationsvermögen der niedrigeren Fettsäuren mit wachsender

Länge der Kohlenwasserstoffkette ansteigt, ist bereits früher an einigen pflanzlichen Objekten festgestellt worden, und zwar von Bouilleinne (1930) an Epidermiszellen von *Tradescantia virginica* und am Blattgewebe von *Allium*, von Collander und Mitarb. (1931) an Epidermiszellen von *Rhoeo discolor*, sowie von Hill (1932) an den Zellen eines Leuchtbakteriums. Unter tierischen Objekten lieferten wenigstens rote Blutkörperchen (Jacobs 1926—27, Green 1949) und Eizellen von *Arbacia* (Stewart 1931) analoge Ergebnisse, wenn auch nach Green das Permeationsvermögen der Fettsäuren Blutkörperchen gegenüber von der Valeriansäure aufwärts wieder abnimmt.

Es scheint somit, dass die Hefezellen hinsichtlich ihrer Permeabilität für Säuren im Wesentlichen mit den Zellen der höheren Organismen und der Bakterien übereinstimmen.

Summary

The permeation of some organic acids into yeast cells has been studied by addition of a solution of the acid to a suspension of yeast cells in water and by then watching the pH-changes occurring in the suspension during the first minute after the addition of the acid. Assuming that the rise of the extracellular pH-value is due to the permeation of the acid into the cells it is possible to estimate the value of the permeation constant of the acid.

In this way it was found that the permeation constants of the straight-chain fatty acids are approximately proportional to their relative ether solubilities, except that smaller molecules have a somewhat greater permeation power than their solubility properties alone would imply.

Acids possessing branched hydrocarbon chains have smaller permeation constants than one would expect on account of their ether solubility and molecular weight.

In solutions of hydroxy fatty acids or of dibasic acids no rise of the extracellular pH-value during the first minute after the addition of the acid could be detected.

The permeability properties of baker's yeast stages cultivated under different conditions were only slightly different. Neither did the permeability of the cells change much during the different growth phases. The cells of brewer's yeast were found, at an average, some 2—3 times more permeable than those of the commercial baker's yeast.

Wir sind Herrn Sakari Rihtniemi zu Dank verpflichtet für seine Mithilfe bei der Durchführung der experimentellen Arbeit.

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Der Einfluss des Lichtes auf die Bildung von Licht- und Schattenblättern der Buche, *Fagus silvatica*

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In den meisten pflanzenphysiologischen Lehrbüchern kann man bei der Besprechung von Licht- und Schattenblättern der Buche lesen, dass der Unterschied im Blattbau durch die Beleuchtungsstärke hervorgerufen wird, die zur Zeit der Blattanlage, d.h. also im Jahr vor der Blattentfaltung, auf den Spross einwirkte. Diese Behauptung wurde von Nordhausen 1903 aufgestellt. Seine Versuche wurden mit abgeschnittenen Buchenzweigen ausgeführt. Ob es Schatten- oder Lichtblätter waren, die sich entfalteten, wurde durch Messung der Blattdicke entschieden. Es ist bekannt, dass die Lichtblätter am dicksten sind. Nordhausen plazierte seine Zweige von *Fagus silvatica* in verschiedener Beleuchtung: Sonnenschein, Schatten und in einem dunklen Keller. Es zeigte sich, dass die Lichtknospen bedeutend dickere Blätter entwickelten als Schattenknospen. Später hat Kny (1909) mit seinen „Botanischen Wandtafeln“ zur Ausbreitung dieser Versuchsergebnisse beigetragen. Dagegen hat Holsoe 1951 gezeigt, dass die Blätter von *Liriodendron tulipifera* durch die Beleuchtung geprägt werden, in der sich die Knospen entfalten.

Um die Versuche von Nordhausen einer Prüfung zu unterziehen, wurden Buchenzweige kurz vor der Entfaltung abgeschnitten und teils im vollen Tageslicht, teils im starken Schatten angebracht; die Blätter entfalteten sich nicht bei absoluter Dunkelheit. Es zeigte sich, dass die Beobachtungen von Nordhausen richtig waren. Am dicksten waren diejenigen Blätter, die aus Lichtknospen entstanden, d.h. Knospen, die bei einer Beleuchtungsstärke von mehr als 50 % des vollen Tageslichtes gebildet wurden. Aber die Annahme, dass man aus der Dicke der Blätter Schlüsse auf die Struktur des

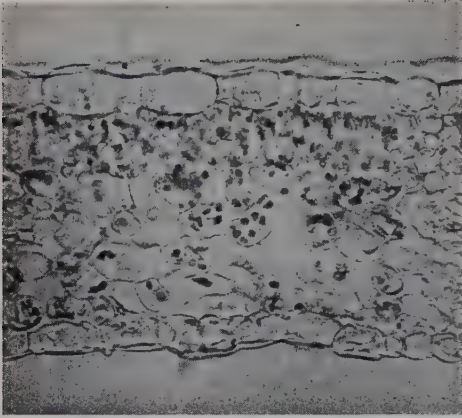


Abb. 1.

Abb. 1. Lichtknospe im tiefen Schatten (0,5 % des vollen Tageslichtes) entfaltet. Das Palisadengewebe ist wie in einem Schattenblatt entwickelt. $\times 421$.

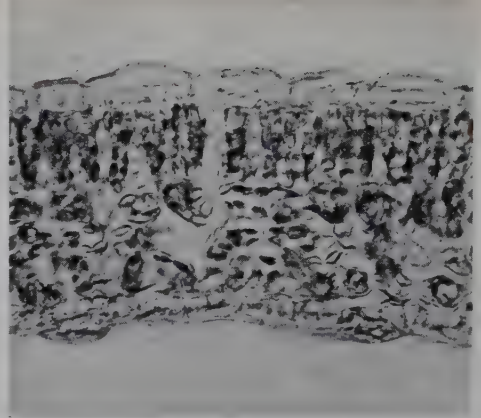


Abb. 2.

Abb. 2. Schattenknospe im tiefen Schatten (0,5 % des vollen Tageslichtes) entfaltet. $\times 421$.

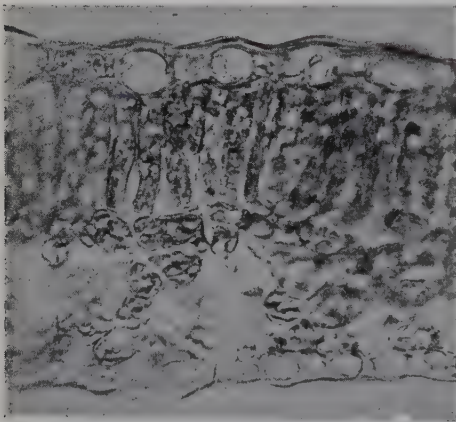


Abb. 3.

Abb. 3. Schattenknospe im vollen Tageslicht entfaltet. Die Palisadenzellen sind lang und stehen dicht, wie es gewöhnlich in Lichtblättern der Fall ist. $\times 421$.

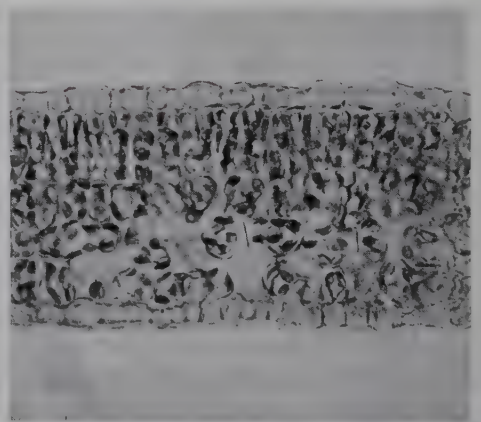


Abb. 4.

Abb. 4. Lichtknospe in 50 % des vollen Tageslichtes entfaltet. Die Palisadenzellen sind nicht so lang und dicht gestellt wie in Abb. 5. $\times 421$.

Assimilationsparenchyms ziehen könnte, war nicht richtig; denn es zeigte sich, dass ein Blatt aus einer Lichtknospe hervorgegangen, aber im starken Schatten entfaltet, ein schwach entwickeltes Palisadengewebe zeigte, d.h. ein Palisadengewebe mit kurzen Palisadenzellen, und nicht wie vermutet

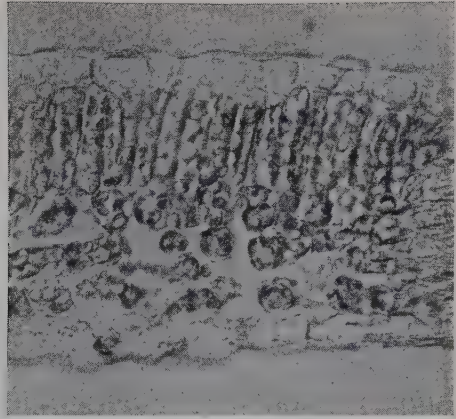


Abb. 5. Lichtknospe im vollen Tageslicht entfaltet. Die Palisadenzellen sind lang und dicht gestellt. $\times 421$. Vgl. Abb. 3.

die Struktur eines Lichtblattes aufwies. Es war also nicht durch die Beleuchtung des vorhergehenden Jahres geprägt worden, sondern durch die geringe Beleuchtungsstärke, die zur Zeit der Blattentfaltung herrschte, vgl. Abb. 1.

Um diese Tatsache näher zu untersuchen, wurden sowohl Licht- als auch Schattenknospen nach Entfaltung unter verschiedener Beleuchtung untersucht. Entfaltet sich ein Blatt einer Schattenknospe im Schatten, dann bekommt das Assimilationsgewebe die für Schattenblätter charakteristische Struktur: kurze Palisadenzellen mit zugespitzter Facon, wie auf Abb. 2 ersichtlich ist. Entfaltet sich ein Blatt in stärkerer Beleuchtung, werden die Palisadenzellen länger und dichtständiger. Im Licht eines Gewächshauses, aber geschützt gegen direkte Sonne, zeigten die Palisadenzellen gleiche Länge und Dichte wie bei einem Lichtblatt, vgl. Abb. 3. Blätter von Lichtknospen zeigten das gleiche Verhalten: Wenn sie sich im vollen Tageslicht entfalteten, hatten sie lange und dichtgestellte Palisadenzellen (Abb. 5) aber in schwächerer Beleuchtung entfaltet waren die Palisadenzellen kürzer und nicht so dichtständig (Abb. 4).

Um Blätter der Licht- und Schattenknospen, die sich bei gleicher Beleuchtung in situ entfaltet hatten, vergleichen zu können, wurden einige Zweige eines im freien wachsenden Baumes entfernt, und zwar so, dass Licht- und Schattenknospen der restierenden Zweige die gleiche Beleuchtung erhielten. Aus Abb. 6 und 7 ersieht man, dass das Palisadengewebe die gleiche Entwicklung zeigt.

Die Lichtknospe ist bedeutend grösser als die Schattenknospe. Aber dieser Unterschied erklärt sich nicht nur aus der Tatsache, dass Blattanlagen und Knospenschuppen bedeutend grösser sind als in Schattenknospen, sondern auch dadurch, dass die Anzahl der Blattanlagen und Knospenschuppen gröss-

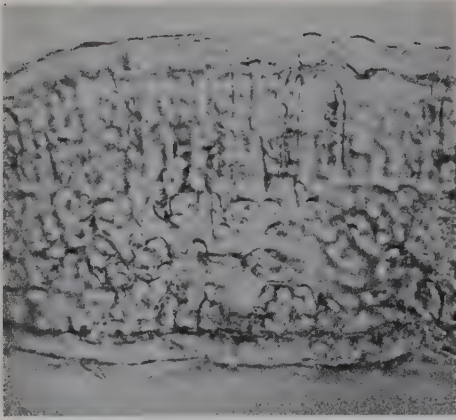


Abb. 6.

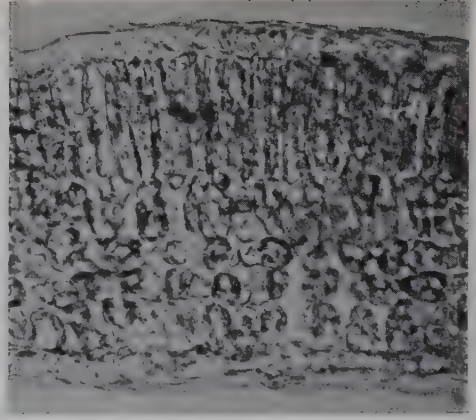


Abb. 7.

Abb. 6—7. Blätter *in situ* entfaltet. Vor der Entfaltung wurden so viele Schatten gebenden Zweige entfernt, dass auch die Schattenknospen sich im vollen Tageslicht entfalteten. Abb. 6 Blatt aus einer Lichtknospe, Abb. 7 Blatt aus einer Schattenknospe entwickelt. $\times 421$.

ser ist. Die ausgeprägte Lichtknospe bei Buchen hat ca. 24 Schuppen und ca. 6 Blätter, während die ausgeprägte Schattenknospe nur ca. 13 Schuppen und 3 Blätter hat. Dazwischen gibt es alle Übergangsformen. Es muss angenommen werden, dass die Grösse der Stoffproduktion des Stützblattes die Grösse der Knospe beeinflusst, selbst wenn man nicht davon absehen kann, dass auch andere Faktoren, so z.B. die Plazierung der Knospen am Zweige, von Bedeutung sein kann.

Dass die Blattanlagen der Licht- und Schattenknospen im Ruhestadium gleiche Dicke haben, bedeutet nicht, dass die daraus hervorgehenden Blätter auch die gleiche Dicke haben müssen. In den Blattanlagen liegen die Blätter zusammengepresst, vergleichbar mit einer Luftmatratze. Im zusammengefalteten Zustand sind sie gleich dick, im aufgeblasenen Zustand wird die grössere auch die dickere sein.

Da keine Zellteilung während der Blattentfaltung stattfindet, ist die Grösse der Blattanlage entscheidend für die Grösse des ausgesprungenen Blattes. Das Zellgewebe in der Blattanlage ist dort, wo sich später das Assimilationsgewebe bildet, im Ruhestadium nicht voll entwickelt; Abb. 8 und 9 zeigen die Blattanlage einer Licht- und Schattenknospe. Die endliche Struktur des Blattes entsteht nach dem Blattausbruch. Abb. 10 zeigt ein Blatt während der Entfaltung im Lichte; es hat noch nicht die endliche Struktur.

Im Gegensatz zum Assimilationsgewebe ist die Struktur im Leitbündel und

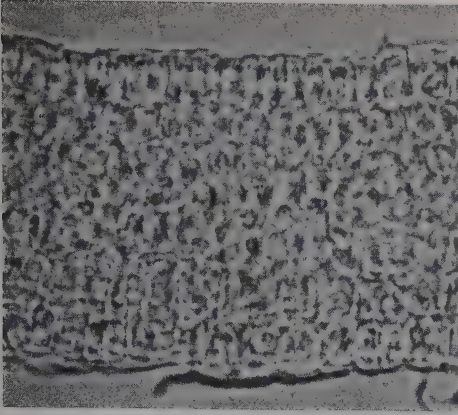


Abb. 8.

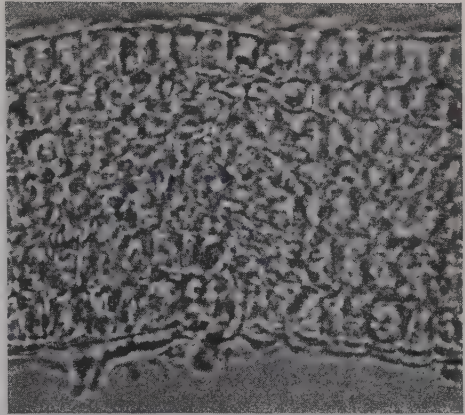


Abb. 9.

Abb. 8. Die Blattanlage einer Schattenknospe. Auch hier ist wie in Abb. 9 das Gewebe nicht differenziert. $\times 680$.

Abb. 9. Die Blattanlage einer Lichtknospe. Das Gewebe ist noch nicht differenziert. $\times 680$.



Abb. 10.

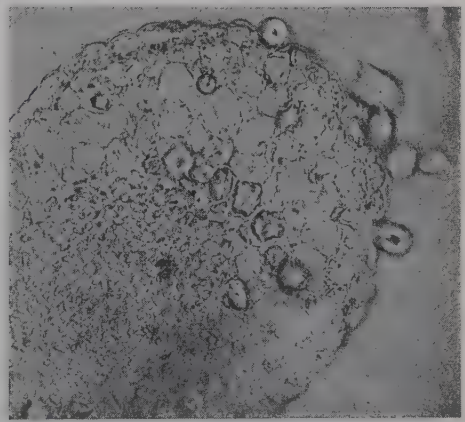


Abb. 11.

Abb. 10. Ein eben entfaltetes Blatt. Das Assimilationsgewebe hat noch nicht die endliche Struktur. $\times 521$.

Abb. 11. Leitbündel im Blatt einer Knospe. Das Zellgewebe des Leitbündels ist schon vor der Blattentfaltung differenziert. $\times 421$

in der Epidermis schon in den Knospen entwickelt, Abb. 11. Auch die Epidermiszellen sind schon in diesem Stadium deutlich abgezeichnet, vgl. Abb. 8 und 9.

Der Blattbau in den Licht- und Schattenknospen ist gleich; die Blätter

sind also nur in der Flächengrösse unterschiedlich. Es treten zwei verschiedene Gruppen von Eigenschaften in den Blättern auf. Erstens die Eigenschaften, die voll entwickelt sind, bevor die Blätter sich entfalten: Leitbündel und Epidermis und Grösse der Blattanlage; zweitens diejenigen, die in den Knospen nicht die endliche Struktur haben und darum die Möglichkeit besitzen sich den Umweltfaktoren anzupassen, in diesem Falle das Assimilationsgewebe, das sich je nach der bei der Entfaltung herrschenden Beleuchtung zu einem Assimilationsgewebe eines Licht- oder demjenigen eines Schattenblattes entwickelt.

Die Versuche von Nordhausen zeigten, dass die Dicke der Blätter von der Beleuchtung abhängig ist, die bei der Anlage des Blattes auf die Knospe einwirkte, d.h. also der Beleuchtung in dem Jahre vor der Blattentfaltung. Die jetzt durchgeführten Versuche zeigen aber, dass die Struktur des Assimilationsgewebes abhängig ist von der Beleuchtung, die zur Zeit der Entfaltung auf das Blatt einwirkt. Die Ursache für Nordhausen's Fehlschluss muss darin gesehen werden, dass damals diese prinzipiell verschiedenen Eigenschaften, die Dicke des Blattes einerseits und die Ausbildung des Assimilationsgewebes andererseits, nicht klar unterschieden wurden.

Summary

The size of the leaves of European Beech, *Fagus silvatica*, and the structure of their epidermis and vascular bundles are determined by the light intensity to which the bud is exposed the year before the unfolding of the leaves. The structure of the mesophyll, on the contrary, especially the palisade tissue, is influenced by the light intensity in which the leaves unfold.

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Effect of Dinitrophenol and Glucose on Oxygen Uptake of Wheat Root Tissue

By

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(Received May 20, 1959)

Suitable concentrations of 2,4-dinitrophenol (DNP) cause considerable respiratory stimulation in excised wheat roots, both when the roots are starved and when exogenous substrate is supplied (Stenlid 1949, Eliasson and Mathiesen 1956). DNP is thought to affect the respiratory processes by uncoupling phosphorylation from oxidation (Läties 1957, Eberhardt 1958). According to this concept DNP will increase oxygen uptake only in tissues where the rate of phosphate turnover is limiting the oxidation. If the concept is correct, a consequence would be that it is the phosphate turnover and not the availability of readily respirable substances that controls the oxidation rate in the starving root tissue investigated (Eliasson and Mathiesen 1956).

Glucose supplied to starving wheat root tissue stimulates the respiration. If the rate of phosphate turnover is the controlling factor in such tissue, it is reasonable to conclude that this stimulation is due to the increased rate of phosphate turnover caused by glucose. Biochemically the evidence for such a mechanism is strong. Glucose in the cell metabolism may function both directly as a phosphate acceptor (cf. Krebs 1957) and as material utilized in synthetic processes connected with conversion of energy-rich phosphate donors (ATP) to phosphate acceptors (ADP). Working with ascites tumor cells, Chance and Hess (1959) obtained experimental evidence that the increase of the respiratory rate caused by glucose addition is effectuated by an increase in the intracellular concentration of ADP and not by the increase of available respiratory substrates.

The experiments reported in this paper have been carried out to provide a more complete picture of the effect of glucose on the respiratory response to DNP in root tissue.

Methods

Roots of wheat seedlings (Weibull's Eroica) grown under sterile conditions according to methods described earlier (Eliasson 1955) constituted the experimental material. The seedlings were germinated in Petri dishes for two days and transferred to a nutrient solution of the composition used by Eliasson and Mathiesen. The roots were grown in this solution for three days at 22°C in the dark. The length of the roots was then about 8 to 10 cm. The terminal 4 cm of the root tips were not utilized in the experiments. The remaining parts were dissected in 1 cm pieces. After material sufficient for all the experiments of the day had been prepared, the root pieces were mixed at random and distributed in Warburg flasks. The respiratory measurements were started about three hours after cutting the roots. The oxygen uptake was determined by standard Warburg technique as described by Umbreit et al. The rate of shaking used was 125 oscillations per minute. In order to obtain approximately equal oxygen consumption per flask in the controls, the number of root pieces in each flask was varied from 30 pieces at 35° to 80 pieces at 15°C. The fresh weight of the root material was determined after the respiratory measurements. In the respiratory flasks the root pieces were suspended in 2 ml 0.02 *M* phosphate buffer, pH 6.7. Glucose (0.05 *M*) sometimes was added to this solution before the beginning of the measurements. In other experiments it was added from the sidearm like DNP subsequent to the determination of the oxygen uptake for 2 hours. The glucose solutions were prepared immediately before use by dissolving a weighed amount of glucose in a sterile buffer solution.

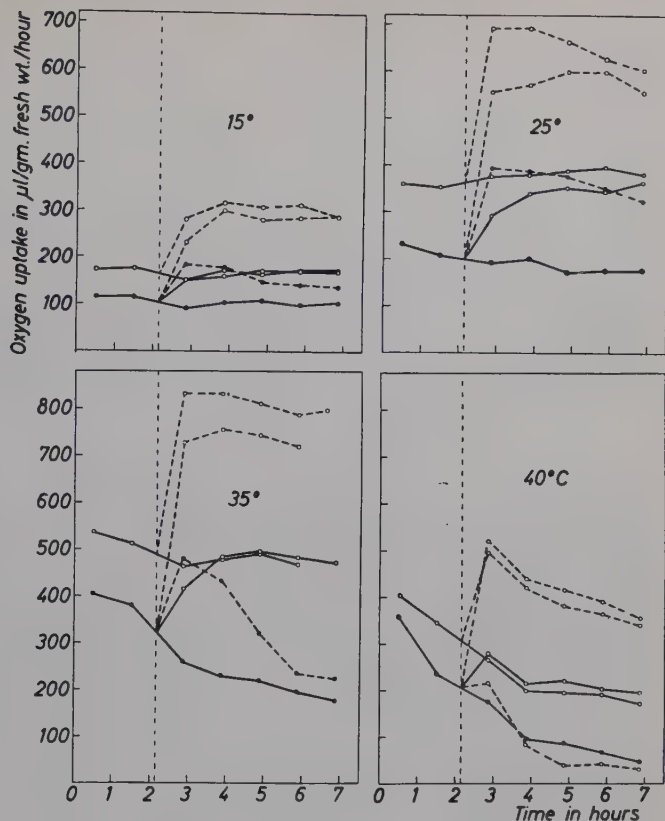
Only mature parts of the roots were used in this investigation, since this tissue was considered a reasonably uniform material with high respiratory responses to both DNP and glucose. Root tips, on the other hand, have been found rather complex in their physiological reactions. Thus, Jensen (1955) found a great variation regarding the response to glucose supply in different parts of the meristem of *Vicia faba*. The response to both glucose and DNP varies greatly in the root tips of wheat (Karlsson and Eliasson 1955, Eliasson and Mathiesen 1956). The fact that the diffusion of oxygen through the tissue may limit the respiration in the meristem at higher temperatures (Berry and Norris 1949) is another reason for avoiding the tips in an investigation involving respiration at different temperatures.

Results

The time course of the oxygen uptake under different conditions used is shown in Figure 1. Similar series of experiments have also been carried out at 20 and 30°C. Values derived from these experiments have been included in Figure 2, where temperature curves representing different treatments are shown.

The relative increase of the respiration produced by glucose and DNP is rather uniform at temperatures ranging between 15 and 35°. Only at 40° is there a diverging response to DNP in the absence of exogenous glucose (Figure 1). However, the most noticeable difference between the rates of

Figure 1. *The effect of glucose and DNP on the rate of oxygen uptake of wheat root tissue at 15, 25, 35, and 40°C. The root pieces were suspended in 0.02 M phosphate buffer, pH 6.7. Subsequent to respiratory determinations for 2 hours the solutions in the side-arms were added. — Filled circles: no glucose. Open circles: 0.05 M glucose. Full lines: no DNP. Dashed lines: 5×10^{-5} M DNP. Each point represents the mean of at least four determinations.*



respiration at low and high temperatures is shown by the time courses of the endogenous respiration. The gradual decrease in oxygen uptake is strongly enhanced at the higher temperatures in tissue not supplied with exogenous substrate. This time-dependent decrease lowers the temperature optimum during the experiment (Figure 2). The curves indicate that the processes effectuating low respiration in starving root tissue are accelerated at higher temperatures. In the presence of glucose, however, the optimum is not affected until the temperature exceeds 35° . Glucose still has a strong stimulative effect on the oxygen uptake at 40° . The stimulative effect of DNP on the oxygen uptake is great both at 35 and 40° provided exogenous substrate is supplied. The fact that DNP and glucose stimulate respiration also at these temperatures indicates that heat inactivation of respiratory enzymes is not the dominant cause of the time-dependent respiratory decrease in this temperature range. The low endogenous respiration is likely to be produced by a control mechanism working in the cells.

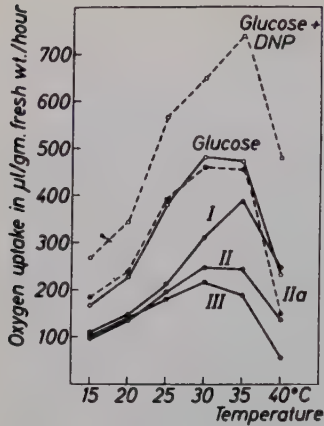


Figure 2. Temperature curves for oxygen uptake in wheat roots. The curves with filled circles: no glucose. *I* 0 to 2 hours, *II* and *II a* 2 to 4 hours, *III* 5 to 7 hours after the beginning of the experiment. *II a*: 5×10^{-5} M DNP. The curves representing "Glucose" and "Glucose + DNP" show the oxygen uptake 2 to 4 hours after beginning of the experiment. The curves are partly based on the same experiments as those of Figure 1. Other details as in Figure 1.

In the presence of DNP there is generally a decrease of the oxygen uptake with time. This decrease is obtained also in suboptimal concentrations of DNP as shown by the curves in Figures 3 and 4. These curves show the oxygen uptake at different concentrations of DNP both during the first two hours and during the period 3 to 5 hours after addition of DNP. This time-dependent respiratory decrease is accelerated at higher DNP concentrations. Thus, there is no sharp borderline between stimulative and inhibitive concentrations

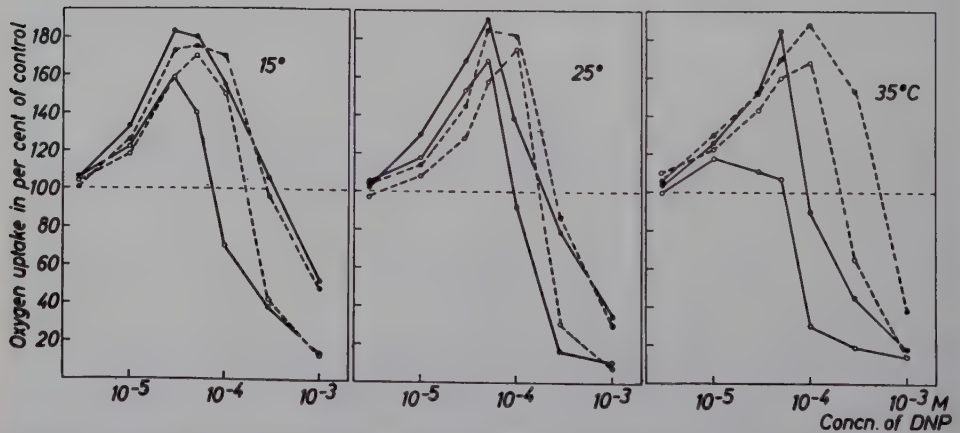
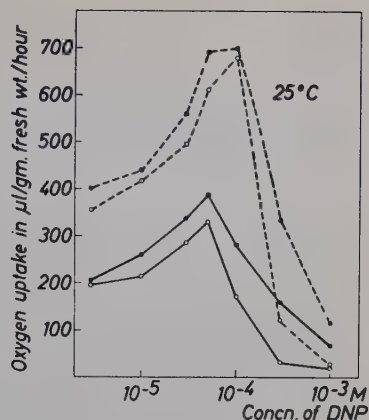


Figure 3. Respiratory response of wheat root tissue to different concentrations of DNP at 15, 25, and 35°C. Full lines: no glucose. Dashed lines: 0.05 M glucose added before the beginning of the respiratory determinations. DNP was added after determination of oxygen uptake for 2 hours. Filled circles represent the oxygen uptake during the 2 hours following addition of DNP, open circles during the period 3 to 5 hours after DNP addition. The oxygen uptake in parallel experiments under the corresponding intervals has been used as control values. Each point represents the mean of four determinations.

Figure 4. Oxygen uptake in wheat root tissue in the presence of DNP at 25°C. The points represent absolute values. Other details as in Figure 3.



of DNP. Intermediate concentrations cause a stimulation in the beginning of the experiment, whereas they inhibit the respiration later on (cf. Stenlid 1949).

The maximal stimulation of the oxygen uptake produced by DNP is fairly equal when relative values are considered regardless of whether glucose is present or not (Figure 3). The absolute values of oxygen uptake, however, are much lower in tissue not supplied with exogenous substrate as exemplified by the curves of Figure 4. This is also expressed by the difference between the respiratory stimulation obtained by supply of DNP only and that obtained by supply of both DNP and glucose to starving root tissue (Figure 1). It may be concluded that the maximal oxidation capacity of starving tissue is not mobilized by DNP.

The time-dependent respiratory decrease in the presence of 5×10^{-5} M DNP is more rapid when no glucose is supplied (Figure 1). This difference in the response to DNP is most pronounced at the higher temperatures used. Evidently the sensitivity of starving tissue to the inhibitory effect of DNP is higher than that of tissue supplied with glucose. This conclusion is also supported by the fact that the concentration of DNP causing maximal stimulation of respiration in starving roots is lower than that effective in the presence of glucose (Figure 3). This is the case especially at higher temperatures.

Discussion

There are strong reasons for assuming that the concentration of the phosphate acceptor ADP is the factor normally controlling the rate of cell respiration (Hunter 1951, Laties 1957, Eberhardt 1958). Consequently, any change

in the rate of oxygen uptake may be suspected to be a result of an altered ADP concentration. The decrease in oxygen uptake found in excised plant tissue not supplied with carbohydrate substrate may thus be explained by the fact that the phosphate acceptor concentration is low in such tissue due to impaired synthesis. Since it is well established that the respiratory stimulation obtained by DNP addition is due to the fact that DNP interferes with the phosphorylation, the experiments provide good evidence that the phosphate turnover really is limiting respiration in such tissue. There are strong reasons for believing that the increase in oxygen uptake caused by glucose addition is not primarily the result of a higher concentration of respirable substrates in the cells, but it depends on an increased conversion of ATP to ADP. This question has recently been discussed by Chance and Hess (1959).

As a consequence of the control mechanism discussed, the varying respiration rate at different temperatures must be associated with corresponding changes in the rate of phosphate turnover. The present results indicate that the low Q_{10} -values obtained at high temperatures may not be explained as an exclusive result of destructive processes affecting the respiratory mechanism. However, a closer investigation of the effect of temperature on the phosphate turnover is necessary if a more detailed picture of the part played by the phosphorylation in the control of the respiration at higher temperatures is to be obtained.

Glucose addition causes a change in sensitivity to the inhibitive effect of DNP on oxygen uptake. The DNP inhibition of oxidative processes has occasionally been ascribed to a direct inactivation of enzymes by DNP (cf. Simon 1953). This hypothesis, however, makes it difficult to explain the lower sensitivity to DNP inhibition in the presence of exogenous glucose. Another hypothesis is that the availability of ATP may be the limiting factor for respiration in the presence of DNP (Potter and Rechnagel 1951, Eliasson and Mathiesen 1956). This hypothesis is founded on the fact that it is probable that presence of ATP is necessary for the normal functioning of the mitochondria (cf. Laties 1953). Thus the gradual decrease of the oxygen uptake in the presence of DNP should be the result of progressive damages to the oxidative mechanism of the mitochondria that occur at deficiency of ATP. The lower sensitivity to DNP inhibition in the presence of glucose may be explained by the fact that phosphorylation associated with glucose fermentation in this case may partially satisfy the ATP demand of the cell. ATP synthesis on the substrate level is thought not to be inhibited by DNP (cf. Anfinssen and Kielly 1954), and it has been found that fermentation is inhibited by DNP only at concentrations higher than those inhibiting oxygen uptake (Beever 1953). Another possibility, which may also explain the higher sensitivity to DNP inhibition in tissue not supplied with glucose, is that the

mobilization of endogenous substrates limits the respiration in the presence of DNP. The primary cause may be ATP deficiency even in this case, as it is known that ATP is necessary for the mobilization of certain substrates (Cross et al. 1949) and that DNP may cause changes in the utilization of substrates (Porter and Runeckless 1956, Stickland 1956).

Summary

The influence of dinitrophenol and glucose on the oxygen uptake in wheat root tissue has been investigated at temperatures between 15 and 40°C.

The maximal stimulation of the oxygen uptake obtained with DNP is about equal regardless of whether glucose is present or not. The concentrations of DNP that produce maximal stimulation are lower without exogenous substrate than in the presence of glucose.

In the presence of DNP there is a time-dependent decrease in oxygen uptake. This decrease is considered to have the same cause as the respiratory inhibition produced by DNP in supra-optimal concentrations.

The results are discussed on the basis of the hypothesis that the phosphate acceptor concentration limits the oxidation rate in the tissue investigated.

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Induction Phenomena in Photosynthesis of Moss Species

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For a number of studies of induction phenomena in photosynthesis the moss species *Polytrichum attenuatum* was used as experimental plant (Vejlby 1958 a, b, 1959). The photosynthesis time curve was here shown to form an induction peak 30 to 60 seconds after the onset of illumination. Previously, van der Veen (1949 a, b), in experiments with leaves of the grass species *Holcus lanatus* and of tobacco plants showed that, in addition, at high light intensities in particular, one or more secondary peaks occur in the time curves. In order to check the possibility of similar phenomena occurring in mosses experiments were made with six pleurocarpous and six acrocarpous mosses (Bryales), and with three species of bog moss (Sphagnales) and three liverworts (Hepaticae).

The results of these experiments demonstrated the existence of a single secondary peak in the time curves of four mosses, *i.e.*, in those of three pleurocarpous species (of genus *Thuidium*) and in that of *Tortula muralis* from the acrocarpous group of Bryales. In the rest of the plants either no secondary peak could be observed, or only a slight shoulder appeared in the curve at the time when one might be expected. In one single case, that of *Helodium blandowii*, no induction peak whatsoever could be found to occur under the given conditions of the experiment.

Material and Methods

The following species were chosen for the main experiments: *Plagiothecium undulatum* (L.) Bruch and Sch., *Pseudoscleropodium purum* (Hedw.) Fleisch., *Helodium blandowii* (Web. and Mohr) Warnst., *Thuidium tamariscinum* (Hedw.) Br. eur

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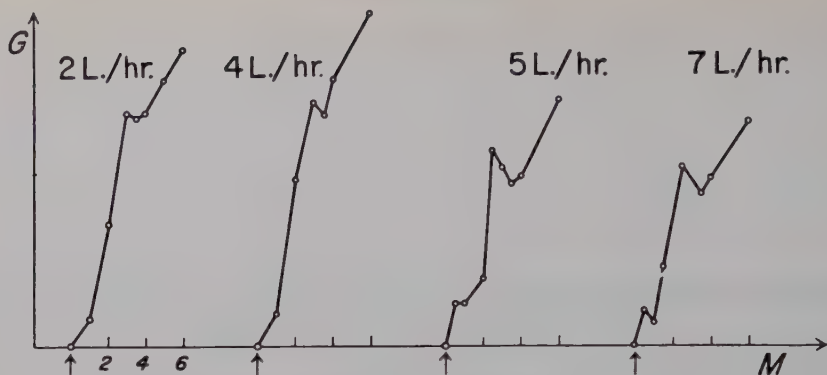


Figure 1. Time curves of photosynthesis for *Thuidium abietinum* in different rates of gas stream, viz. 2, 4, 5, and 7 litres per hour. Upward arrows indicate light on. Abscissa: M = time in minutes. Ordinate: G = galvanometer deflection.

T. philibertii Limpr., *T. abietinum* (Schwaegr.) Br. and Sch., *Dicranum scoparium* Hedw., *D. majus* Turn., *D. undulatum* Br. eur., *Mnium cuspidatum* Hedw., *Polytrichum attenuatum* Menz., *Tortula muralis* Hedw., *Sphagnum parvifolium* Warnst., *S. palustre* L., *S. fimbriatum* Wils., *Fegatella conica* Corda, *Marchantia polymorpha* L. and *Lophocolea cuspidata* Limpr. For classification data see legends of figures 2, 3, and 4.

The sample plants were collected from different localities in Northern Zealand (Sjælland), with the exception of *Thuidium tamariscinum* which came from the southeastern part of the island, and the three liverworts which were obtained from the Botanical Gardens of the University of Copenhagen. As before, the plant material was stored in covered glass dishes in an air thermostat at 5 to 10°C. Sufficient water was supplied to keep the plants fully turgid, and they were exposed to alternating light and dark periods of 18 and 6 hours, respectively (cf. Vejlby 1959).

The experimental arrangement including use of the diaferometer method (the gas thermal conductivity method) was as described before (Vejlby 1958 a). About 0.25 g of plant material (fresh weight) was used for each experiment with exception of the *Sphagnum* experiments where about 1 g was necessary. The plant material was placed in streaming atmospheric air to which had been added 3 vol% of carbon dioxide. The temperature of the gas stream was 15°C. The light intensity was $204 \text{ cal/dm}^2 \cdot \text{hr}$ ($\lambda 400\text{--}700 \text{ m}\mu$) $\sim 7100 \text{ lux}$ (conversion according to Gabrielsen 1948). The light source was a 3000 watt incandescent lamp placed in a running water bath.

While in all previous experiments with *Polytrichum attenuatum* a rate of 3 litres per hour was used for the gas stream, about twice that rate was used in the present investigations. In some preliminary experiments with *Thuidium abietinum*, at 3 litres per hour, it was found that the peak, which from the *Polytrichum* curves was expected to occur after 30 to 60 seconds of illumination, was delayed and occurred only after the plants had been exposed to illumination for 120 to 150 seconds. Various facts were found indicating this delayed peak to be of a somewhat different origin than those observed in *Polytrichum attenuatum* curves. However, an increase of the

rate of the gas stream induced the formation of yet another peak in the *Thuidium* time curve, and this peak appeared at the "usual" time. As an example of the results of the preliminary work concerning the effects of the rate of the gas stream, four time curves with *Thuidium abietinum* are reproduced in Figure 1, the respective rates being 2, 4, 5, and 7 litres per hour. Compare also Figures 5 A and B, where the rates were 4.5 and 6.8 litres per hour, respectively. All the curves reproduced in Figures 1—5 represent typical examples selected from a comprehensive experimental material checked by many repetitions.

Results and Discussion

The main results appear from Figures 2, 3, and 4. The two former represent experiments with pleurocarpous and acrocarpous mosses, respectively, and the latter with *Sphagnum* species and liverworts.

The natural procedure in discussing the results appears to be a comparison of the time curve for *Polytrichum attenuatum* (in Figure 3) with the other 17 time curves in Figures 2, 3, and 4. The *Polytrichum* curve exhibits the characteristic induction peak, the maximum of which occurs after 1 minute illumination. This peak will be termed the sixty seconds peak or primary peak. A corresponding peak may be found in all of the other curves with the exception of *Helodium blandowii* (Figure 2) and perhaps also *Lophocolea cuspidata* (Figure 4); in the latter case, however, a slight shoulder does appear in the time curve after barely 1 minute of illumination. In addition to the sixty seconds peak or primary peak a secondary one occurs in several cases after 2.5 to 4.5 minutes of illumination. This phenomenon is most pronounced in the case of the three *Thuidium* species (Figure 2) and in that of *Tortula muralis* (Figure 3), but shoulder shaped bends are found also in the time curves of *Plagiothecium undulatum* and *Pseudoscleropodium purum* (Figure 2), in *Dicranum scoparium*, *Mnium cuspidatum* and (perhaps) *Dicranum undulatum* (Figure 3) as well as in *Lophocolea cuspidata* (Figure 4).

While according to previous studies (Vejlby 1959) the primary peak must be considered to be a result of the activities of a specific primary acceptor of carbon dioxide, exceeding during the first few minutes of illumination the functions of the CO₂ acceptor (ribulose-diphosphate), which later replaces it and which is generally assumed also to take over the amount of carbon dioxide first bound to the primary acceptor, the secondary peak appears to be of a different type. Massini (1957) showed in experiments with iodoacetamide treated leaves of *Dahlia* that the initial uptake of carbon dioxide (corresponding to the primary peak in the time curves reproduced in the present paper) is inhibited to a considerably lesser extent than the photosynthetic intensity after it has reached a steady state. In a molar concentration of 10⁻² the figures for the inhibition after 2 and 5 hours of treatment are, respectively,

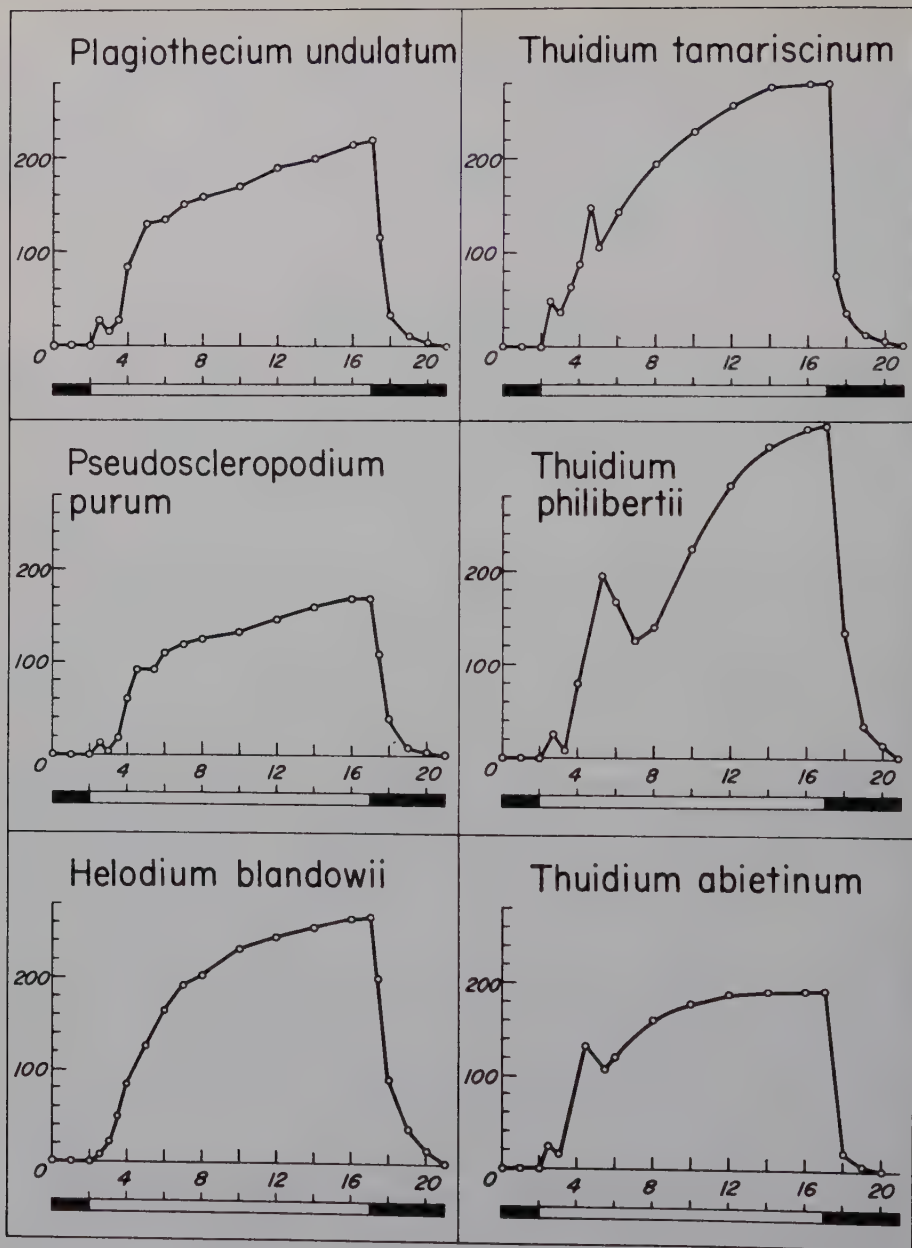


Figure 2. Time curves for six pleurocarpous mosses (Bryales). Abscissa: time in minutes. Ordinate: galvanometer reading in mm. The periods of light and dark are indicated by areas of white and black under the curves.

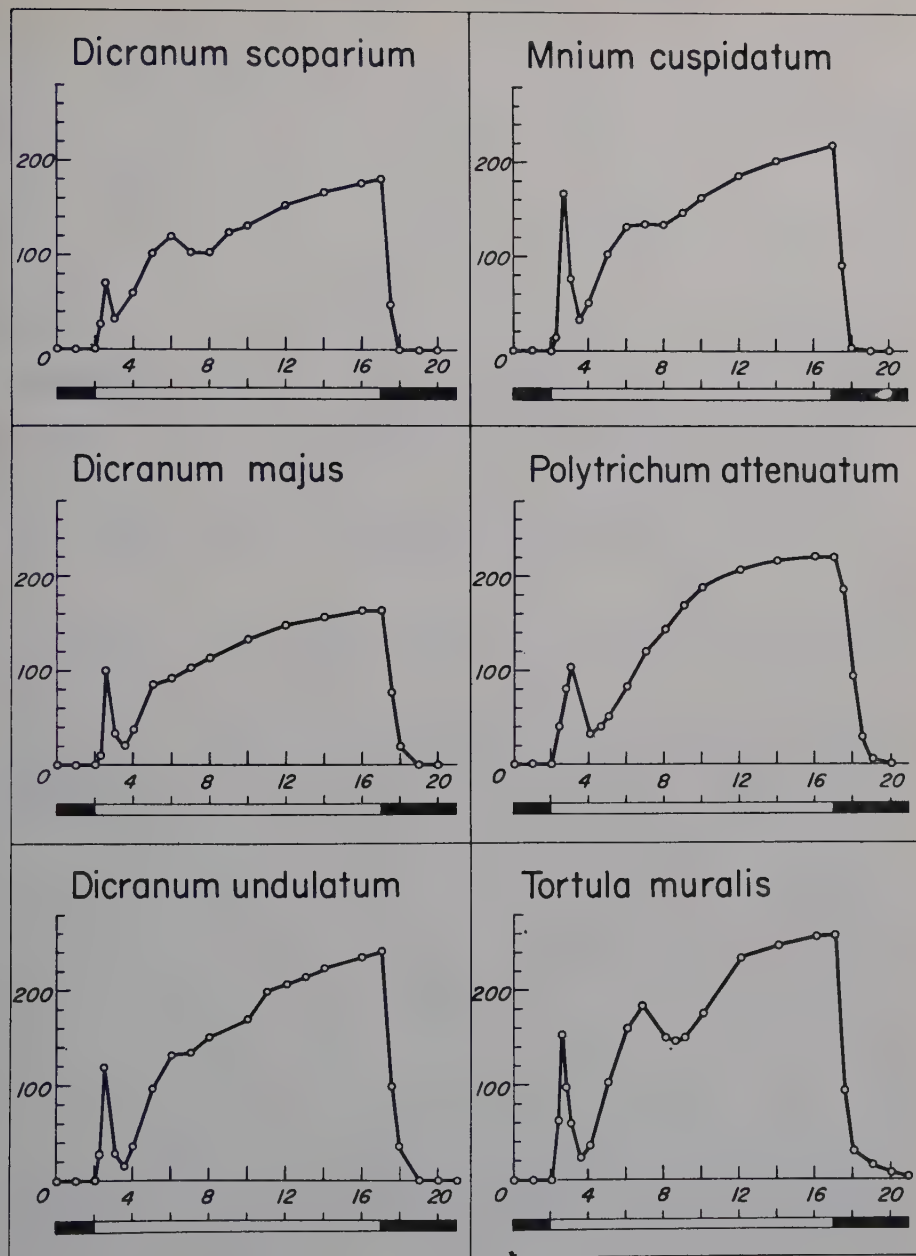


Figure 3. Time curves for six acrocarpous mosses (Bryales).

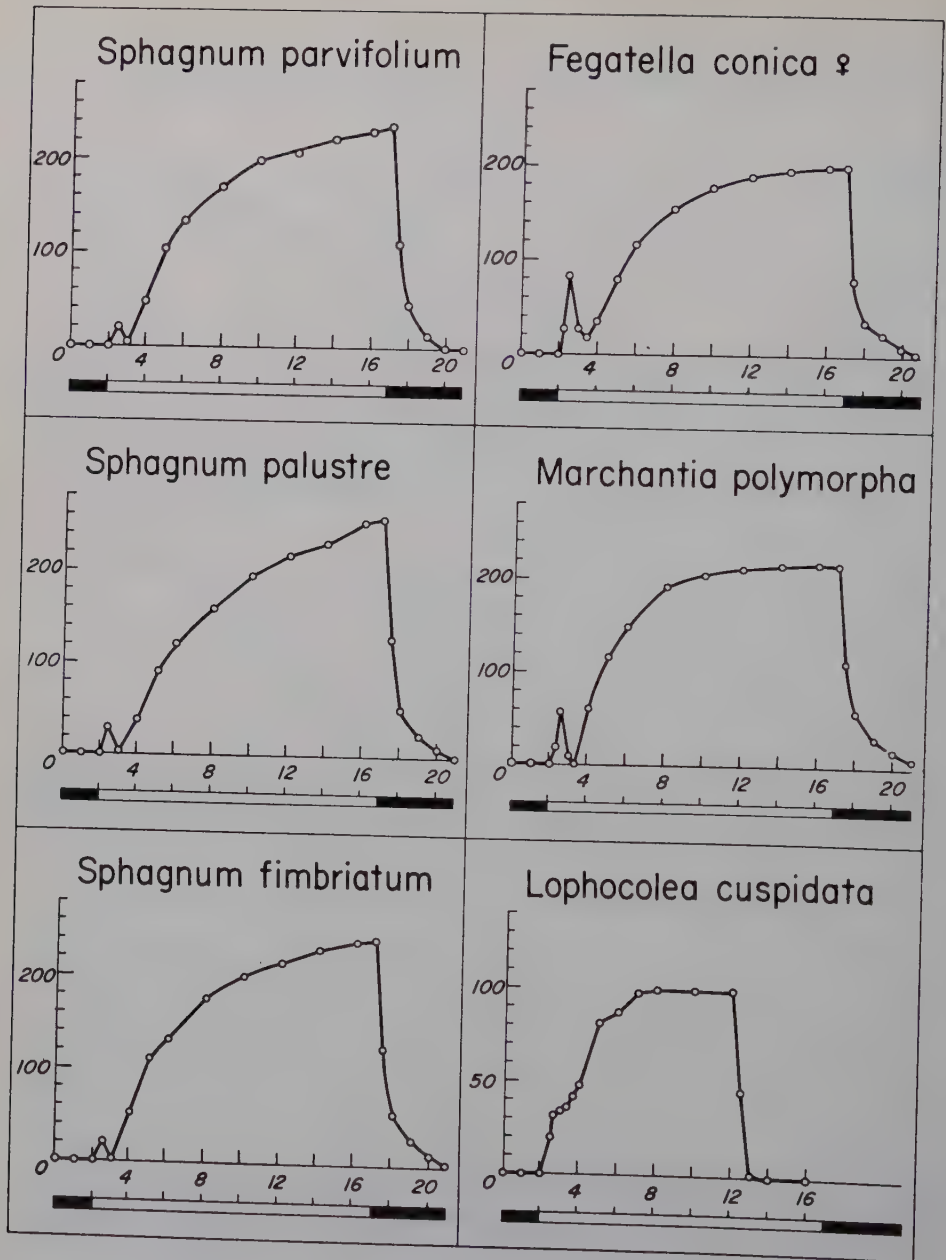


Figure 4. Time curves for three species of *Sphagnum* and for three liverworts (Hepaticae).
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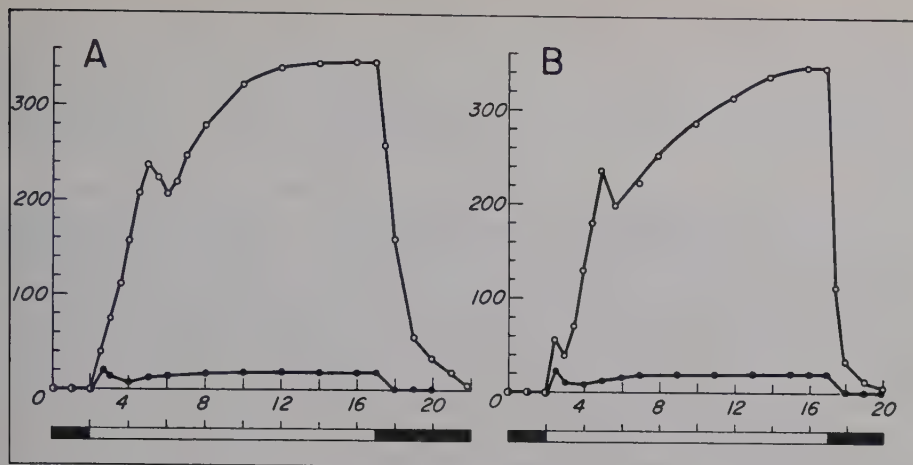


Figure 5. Time curves for *Thuidium tamariscinum*. \circ - \circ untreated plants; \bullet - \bullet same plants after 10 minutes in 10^{-2} M iodoacetamide. Gasflow in A 4.5 litres per hour, in B 6.8 litres per hour.

.95 and 100 % of the photosynthetic steady state intensity, and 0 and 30 % of the initial uptake of carbon dioxide.

Induced by these interesting results iodoacetamide treatment experiments were made with one of the moss species showing a primary as well as a secondary peak in its photosynthesis time curve. When *Thuidium tamariscinum* is treated with a 10^{-2} M solution of iodoacetamide for 10 minutes (all of the plant material being submerged in the solution for the period given) the final photosynthetic intensity (the steady state rate) was inhibited by about 95 % whereas the primary peak was inhibited only by about 60 %, as appears from Figure 5 B. While the primary peak is still very conspicuous in the plants treated with iodoacetamide, the secondary peak evidently disappears under these conditions. It was mentioned above that with the technique used in the present experiments the primary peak in the case of the *Thuidium* species appears only at sufficiently high rates of the gas stream. If the rate is below 5 to 6 litres per hour only the secondary peak occurs in the time curve. But after the plant material has been treated with iodoacetamide, which inhibits the secondary peak, a primary peak becomes distinct, as appears from Figure 5 A.

The failure to demonstrate the existence of the primary peak at low rates of the gas stream, at which the sensitivity of the diaferometer is increased, is probably due to the fact that the front, formed in the gas stream at the change of its composition, is blurred to a greater extent at low rates than at high ones. The blurring takes place while the gas stream is passing the plant mate-

rial. The appearance of the peak in experiments with iodoacetamide treated plants even at low rates, is undoubtedly due to the fact that in this case the total uptake of carbon dioxide is inhibited to such an extent that the change in the uptake of carbon dioxide, represented by the peak, is now relatively high.

It is worth noting that all of the *Sphagnum* species studied in their time curves show only primary peaks, and that all of the *Thuidium* species studied show primary as well as secondary peaks, whereas *Helodium blandowii*, which taxonomically is closely related to *Thuidium* (it is sometimes classified as *Thuidium blandowii*) appears to have no peaks at all in its time curve. Hence the present experiments offer some physiological support for the separation of *Helodium* from *Thuidium*.

Summarizing it may be said that the experiments succeeded in demonstrating the presence of a primary peak in the photosynthesis time curve for 17 out of the 18 species of mosses, liverworts, and *Sphagnum* studied, and that this peak occurs after about 1 minutes of illumination. In the three species of *Thuidium* studied and in *Tortula muralis* the existence of an additional secondary peak was demonstrated. The secondary peak appears to differ in nature from the primary one, in that it, contrary to this, disappears upon the treatment of the plant material with iodoacetamide. Further, there appears to be no connection between the taxonomy of the mosses and the shape of their photosynthesis time curves when greater groups are considered, nor has it been possible to establish any correlation between the latter and the ecology of the moss species studied. Thus *Thuidium tamariscinum* and *Tortula muralis*, which both show primary as well as secondary peaks in their time curves, are mosses characteristic of respectively moist and dry localities.

Summary

The photosynthesis time curves were determined for 18 moss species, 12 from Bryales, 3 from Sphagnales, and three liverworts (Hepaticae). With one exception (*Helodium blandowii*) all of the time curves show a primary peak after about 1 minute of illumination. In 4 of the mosses studied (3 species of *Thuidium* and *Tortula muralis*) an additional, pronounced secondary peak occurs after 2 1/2 to 4 1/2 minutes of illumination. The peaks of the time curves appear to differ in nature, the primary one being much less sensitive to treatment with iodoacetamide than the secondary one.

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Studies on the Nutrition of Forest Tree Seedlings. II

Mineral Nutrition of Spruce

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I. Introduction

A number of laboratory experiments with birch in nutrient solutions have been described earlier (18) and are here supplemented with similar investigations of spruce (*Picea abies*, Karst.). The purpose of these investigations is to explore the nutrient requirements of forest tree seedlings. While very little such work has been done with birch, a great many with spruce have been published. Unfortunately, but a few permit dependable conclusions. As a rule, either the actual nutrient absorption has not been controlled or the investigations lack experimental design. Frequently, too, no attention was paid to secondary effects of the fertilizers that are especially important when lime or potassium salts have been applied. Among effects of this kind may be mentioned high salt concentration in the soil immediately upon the fertilization, changes of pH, changed solubility of nutrients in the soil, and ion antagonism. Due to the negligence of such secondary effects the importance of fertilizers as a source of plant nutrients has often been exaggerated or underestimated.

During recent years a couple of bibliographies comprising silvical nutrient investigations have been published (25, 69). References in the following mainly pertain to work on the nutrient requirements of spruce in controlled experiments. Often the purpose of investigations on the nutritional conditions has been to determine how a nutrient deficiency is to be diagnosed. When studying deficiency in plants mainly two methods have been used,

viz., ocular estimate of deficiency symptoms (Lundblad, 26, Wallace, 68), and foliage analysis (Goodall and Gregory, 14, Lundegårdh, 28).

In spruce disease symptoms for a large number of elements are described and, occasionally, illustrated by pictures (2, 3, 5, 11, 16, 19, 20, 21, 40, 41, 42, 43, 46, 47, 49, 51, 61, 64, 65, 67, 70). Yet, in most of the cases the symptoms appear not to be sufficiently specific to admit an indiscriminate and satisfactory use of this method of diagnosis. The conditions for a correct diagnosis, however, seem to be very good if the observations are combined with a needle analysis. The usefulness of needle analysis for an evaluation of the nutritional status of a tree or a stand has been investigated by several workers (Mitchell, 30, Tamm, 62, 63, 66) and the method has been considered valuable provided the sampling has been uniform (season, position in the crown, and age of needles). In some cases attempts have been made to relate the deficiency symptoms with a special level in the needles of the element in question or with the relationship between the content of this element and that of other elements (5, 19, 24, 25, 62, 64). In this way, the nutrition status of spruce trees that show deficiency symptoms has been determined for some few elements.

Some experiments have also presented values of element contents that correspond to the maximum growth of spruce. Information of this kind, however, is scanty and often controversial. Generally, the conditions in field or in soil culture experiments may be so complicated as to render a correct evaluation of the optimum content of a single element difficult. A number of workers (24, 33, 39, 55, 56, 64) have discussed optimum or "normal" levels of nitrogen. The values vary between 1.2 and 2.7 per cent nitrogen in needles or one-year old shoots of spruce. The discrepancy of the results seems to be caused by differences in the experimental conditions and but in a few cases the results may be considered dependent entirely on the nitrogen and its importance as plant nutrient. The optimum content most probable for spruce seems to be approximately 2 per cent. Tamm has obtained results in a recent experiment that confirm this value (personal communication).

Optimum values of phosphorus content, too, have been presented for spruce (24, 34, 55, 56). Data available are quite divergent and the values vary between 0.13 and 0.35 per cent.

The author has found no direct information on the optimum potassium content of spruce needles. In some cases (Björkman, 5, Tamm, 61) the calcium absorption has been considered of importance for the potassium status of spruce. Tamm (61) mentions that a K/Ca-ratio exceeding the value 1 indicates that potassium deficiency is unlikely.

Data available concerning the calcium content of spruce needles or the lime requirements (10, 35, 40, 43, 59) appear to give no reliable information on the

optimum levels, since the secondary effects of lime seem to be of greater importance than its calcium fertilizer effect.

Data that may give an idea of the optimum content of other elements in spruce needles are, to the author's knowledge, generally lacking. However, a number of papers report on problems closely associated with nutrient conditions. Thus the effect of pH has been studied by several workers. Nemec (35) found an optimum pH of approximately 5 for spruce in an experiment with liming of nursery soil, and similar results were obtained by Süchting *et al.* (58, 59). Süchting (57), however, has shown in an experiment series with very acid humus that liming produced a positive effect only when pH(KCl) is as low as ≤ 3.2 . This is ascribed to the balanced nutrient composition of the humus. Leyton (23) has found in a water culture experiment with Sitka spruce seedlings that the optimum pH value seems to range between 4 and 5 for both NO_3^- and NH_4^+ as nitrogen source. The dry weight of the seedlings showed a distinct maximum, but it may be objected against the investigation that the rate of nitrogen application appears to have been proportionally low and that of phosphorus high. There are many indications that the pH-value may vary widely without affecting the growth of plants decisively, if sufficient and well balanced nutrient solutions are supplied (Olsen, 48).

The importance of the mycorrhiza for the nutrient absorption and growth of trees is another question closely associated with the nutrition problems. Björkman (4) has studied the conditions for the development of mycorrhiza in spruce and pine, and a comprehensive bibliography on the problems of mycorrhiza was published in 1956 (7). Melin (29) has summarized investigations dealing with the importance of mycorrhiza for the nutrient absorption. It seems to be shown that mineral substances may be transferred through the mycorrhiza mycelium and that mycorrhiza establishment is impaired when the nutrient content of the substratum is high and balanced. Mycorrhiza, therefore, has been judged important only in soils of low nutrient status. Stimulation effects of mycorrhiza on the development of trees, however, seems to have been shown only for certain special conditions (for instance in prairie soils by, *e.g.*, Hatch, 15) and no long term experiments under well controlled conditions have been carried out as far as the author knows. It has been stated (Björkman, 6, 7) that seedlings intended for planting in field should have mycorrhiza. This, however, seems to be controversial since seedlings subsequent to a rich and balanced fertilization show the best development after planting (Nemec, 34, 44). Very heavily and excessively fertilized (NPK) seedlings, too, show good development and frequently they are more vigorous than seedlings that are poorly or incompletely fertilized (Björkman, 6, 7).

Apparently, the nutrient requirements and growth of spruce at varying supply and absorption of mineral elements still are very incompletely known and the information found in the literature is controversial. The need for better knowledge of this field is conspicuous, not least with respect to nursery production of seedlings.

The effects of various concentrations of mineral elements (N, P, K, Ca, Mg, S, and Fe) on the growth and nutrient absorption of spruce in nutrient solutions are studied in this investigation.

II. Technique

The cultural conditions have largely been similar to those used for birch (18). The temperature during the daily light period, however, has been lowered from 25° to 20°C since Stålfelt (53) has shown that a temperature of 25° is a critical level for spruce at low intensity of light (10 per cent of sun light) because of the rapid retardation of the assimilation above that temperature.

The cultural time has been considerably longer for spruce than for birch (160 days as against 75 days). After half the time the seedlings were culled to the extent that nine were left in each beaker. Solitary seedlings dying in some beakers left but eight at the harvest, and in one case six seedlings only remained (a beaker with the lowest potassium supply).

The experiment has been designed with two replicates in all series; in the iron series, however, one replicate erroneously was given wrong nutrient solutions.

Nutrient Solutions. The nutrient solutions were renewed once a week. The pH-value of the solutions was 4—5 in the beginning but declined somewhat during the course of a week.

As in the case of birch the common treatment contained in all series constitutes

Table 1. *Composition of basic solutions subsequently diluted 1:100 to give the control concentrations.*

Compounds	g/litre	mmol/litre	Used in series
NH ₄ NO ₃	14.3	178.5	All
NaH ₂ PO ₄ ·2H ₂ O	5.05	32.3	N-, P-, and K-series
KH ₂ PO ₄	4.40	32.3	Control, Ca-, Mg-, S-, and Fe-series
KCl	7.13	95.7	"
KCl	9.50	128.0	N-, P-, and K-series
CaCl ₂ ·6H ₂ O	21.9	100.0	All
MgSO ₄ ·7H ₂ O	15.4	63.0	All except Mg- and S-series
MgCl ₂ ·6H ₂ O	14.5	63.0	Mg- and S-series
Na ₂ SO ₄ ·10H ₂ O	20.8	63.0	"
FeCl ₃ ·6H ₂ O	0.50	1.7	All
MnCl ₂ ·4H ₂ O	0.06	0.3	"
H ₃ BO ₃	0.10	1.5	"
ZnCl ₂	0.004	0.03	"
CuCl ₂ ·2H ₂ O	0.005	0.03	"
Na ₂ MoO ₄ ·2H ₂ O	0.0007	0.003	"

Table 2. *Proportions between the elements in the control nutrient solution expressed as ppm.*

Element	ppm	Element	ppm
N	50	Fe	0.93
P	10	Mn	0.17
K	50	B	0.17
Ca	40	Zn	0.02
Mg	15	Cu	0.02
S	20	Mo	0.003

the control. The stock solutions the composition of which are shown in Table 1 were composed to be diluted 1 : 100 for the control. The proportion between various elements in this solution is shown in Table 2. The different supplies of the various elements is accounted for in Tables 3 (A, B) or 4. The chemicals have been of analytical grade and all the water used has been distilled once in a pyrex-glass distiller. The nutrient solutions were diluted in the beginning of the experiment (1 : 4 during the first two weeks and 1 : 2 the following week).

The control solution used for spruce is more diluted than that applied for birch (approx. 1 : 3). The proportions between the elements is also largely the same except in the case of phosphorus (1 : 10).

Sample Treatment, Analytical Methods and Data. The seedlings were cut in tops and roots. The needles were not separated until dry. Otherwise the samples were treated in the same way as that used for birch and the same methods of analysis have been applied (18).

The element content of the needles and roots of the seedlings are consistently expressed in per cent of dry weight. When the contents cited are originally expressed otherwise, the values have been converted. The results of the analysis are summarized in Table 3 A and 3 B.

The statistical processing of the data has been similar to that applied for birch (18) and the standard error, expressed in per cent (ϵ), is presented on the bottom line in each column (Tables 3 (A, B) and 4).

III. Results and Discussions

1. Growing Conditions

The Tables 4 and 5 show that the control solution has been well adapted to spruce and close to optimum under the prevailing conditions. Thus, the total dry weight is highest at the ion strength used in this solution (Table 5) and in all the experiment series the control solution has produced the highest average dry weight (Table 4). Earlier nutrient solution experiments have been carried out with various spruce species or varieties (17, 23, 50, 51). The purpose being different or the investigation preliminary, however, any optimum solution has not been suggested.

The pH-value of the nutrient solutions has been 4—5 during most of the experimental time. Minor variations above and below these values have occurred during short periods the days before and after the renewal of the solutions. An experiment carried out at a pH-value of 5—6 produced considerably inferior growth despite longer time in culture. This may be due to reduced solubility of certain nutrients at these experimental conditions. According to Leyton (23) maximum growth of Sitka spruce is obtained at a pH-value of 4—5, the very same range as that used in the present investigation.

Thus, it has been shown that the nutrient solutions were composed to exclude any serious interference by secondary influence of the nutrient solution on the effect of single elements. Yet, the solution is considered only as a medium to produce seedlings of a certain physiological nutrient status. This status is described by means of the growth and the content of various elements in needles and roots.

The roots of the seedlings lack mycorrhiza in all cases. Since several workers claim mycorrhiza to be of great importance for the development of forest trees under nutrient deficiency (4, 6, 7, 15, 29), it is possible that the growth curves obtained in this investigation are special cases within the sub-optimum range. However, the influence of mycorrhiza on the nutrient absorption is subordinate in this context since the roots have been in a very intimate contact with the nutrients and since the nutrient absorption is described on the basis of the element content of needles and roots. It is possible, however, that the absence of mycorrhiza affects the growth by the failure of other effects to appear (*e.g.*, changed carbohydrate status and failing effects of substances exuded from the fungus).

Thus, it may be emphasized that the results obtained at an investigation of this kind cannot directly be applied in the field. This may be ascribed not only to the absence of mycorrhiza, but mainly to the relatively low intensity of light in the chamber. Far reaching conclusions on the applicability may not be drawn until comparisons with seedlings grown under field conditions are possible. Some inferences may be made even at the present, but field experiment data now available are scanty and often independable. However, a very good accordance is noticeable when data are comparable.

2. Morphological Effects

A rather continuous growth has been attained in all solutions with sufficient nutrient supply. Occasionally, however, a certain node formation has occurred 1—3 times during which a reduction of the growth rate has been

Table 3 A. *Element content of needles. Control figures in italic type.*

Element	Supply, ppm	Dry weight of needles, g/beaker	Element content, % of dry weight						
			N	P	K	Ca	Mg	S	Fe
N	0.5	0.44	0.7	0.27	0.78	0.37	0.13		0.005
	5.0	1.21	1.0	0.24	0.75	0.30	0.11		0.005
	15	2.21	1.7	0.25	0.94	0.26	0.13		0.006
	50	2.70	2.1	0.23	0.93	0.19	0.11		0.007
	150	2.43	2.3	0.25	0.96	0.21	0.12		0.007
	300	2.27	2.4	0.25	1.02	0.25	0.13		0.010
P	0.1	0.50	2.5	0.05	1.19	0.35	0.21		0.097
	1.0	2.52	1.7	0.11	0.75	0.21	0.13		0.006
	3.0	2.68	1.9	0.24	0.81	0.24	0.13		0.006
	10.0	2.70	2.1	0.23	0.93	0.19	0.11		0.007
	30	2.61	2.0	0.25	0.85	0.22	0.12		0.007
	60	2.67	2.2	0.32	0.92	0.20	0.12		0.007
K	0.15	0.44	2.1	0.32	0.26	0.29	0.21		0.007
	1.5	1.68	2.2	0.27	0.31	0.27	0.27		0.006
	15	2.41	2.2	0.23	0.74	0.23	0.13		0.007
	50	2.70	2.1	0.23	0.93	0.19	0.11		0.007
	150	2.42	2.3	0.26	1.09	0.25	0.14		0.009
	300	2.33	2.2	0.23	1.34	0.21	0.12		0.007
Ca	0.12	1.45	2.3	0.26	1.08	0.02	0.13		0.009
	1.2	2.20	2.1	0.23	1.87	0.02	0.10		0.006
	12	2.46	2.1	0.26	0.98	0.12	0.13		0.007
	40	2.70	2.1	0.23	0.93	0.19	0.11		0.007
	120	2.55	2.1	0.22	0.87	0.50	0.11		0.006
	(360 ¹)	1.79	1.9	0.21	1.03	0.99	0.09		0.005)
Mg	0.05	0.81	1.8	0.32	1.91	0.28	0.02		0.005
	0.15	1.61	1.8	0.29	1.40	0.26	0.02		0.005
	1.5	1.80	2.2	0.26	1.06	0.29	0.07		0.010
	15	2.70	2.1	0.23	0.93	0.19	0.11		0.007
	45	2.23	2.0	0.22	0.98	0.21	0.21		0.005
S	0	0.71	1.7	0.22	1.12	0.45	0.20	0.11	0.006
	2.0	1.84	2.1	0.22	0.88	0.25	0.12	0.13	0.006
	6.0	2.28	2.1	0.24	1.06	0.27	0.15	0.16	0.008
	20	2.70	2.1	0.23	0.93	0.19	0.11	0.18	0.007
Fe ¹	0	0.77	1.7	0.28	0.93	0.28	0.15		0.002
	0.02	2.60	1.6	0.24	1.02	0.27	0.13		0.003
	0.2	2.65	2.0	0.24	1.06	0.24	0.13		0.006
	1.0	2.70	2.1	0.23	0.93	0.19	0.11		0.007
± s %		8.0	7.9	8.8	6.9	13.0	12.8	(7.2)	18.7

¹ No duplicates.

observed. At severe deficiency, some seedlings have established terminal buds which, however, have failed to develop further.

Deficiency symptoms have occurred in all the nutrient series at the lowest (P, K, Ca, S) or the lowest two rates of supply (N, Mg, Fe). These symptoms

Table 3 B. *Element content of roots.* Control figures in italic type.

Element	Supply, ppm	Dry weight of roots, g/beaker	Element content, % of dry weight						
			N	P	K	Ca	Mg	S	Fe
N	0.5	0.43	0.91	0.42	0.98	0.52	0.13		0.66
	5.0	1.04	1.42	0.53	1.64	0.22	0.12		0.27
	15	1.00	2.35	0.52	1.75	0.22	0.10		0.18
	50	1.01	2.71	0.41	1.06	0.22	0.08		0.30
	150	0.94	2.96	0.46	1.32	0.13	0.02		0.24
	300	0.77	3.63	0.52	1.36	0.16	0.04		0.28
P	0.1	0.43	1.98	0.10	0.89	0.22	0.03		0.36
	1.0	1.10	2.23	0.22	1.26	0.14	0.07		0.16
	3.0	1.04	2.57	0.38	1.10	0.14	0.06		0.14
	10.0	1.01	2.71	0.41	1.06	0.22	0.08		0.30
	30	1.05	2.93	0.57	1.24	0.14	0.06		0.12
	60	0.95	2.60	0.48	0.97	0.14	0.07		0.22
K	0.15	0.12	3.03	0.62	0.36	0.20	0.10		0.59
	1.5	0.79	2.78	0.40	0.39	0.13	0.08		0.28
	15	0.97	2.40	0.42	1.04	0.12	0.10		0.28
	50	1.01	2.71	0.41	1.06	0.22	0.08		0.30
	150	0.97	2.53	0.44	1.51	0.16	0.13		0.22
	300	0.76	2.86	0.46	1.60	0.13	0.13		0.28
Ca	0.12	0.51	2.43	0.41	0.74	0.04	0.10		0.35
	1.2	0.75	2.46	0.37	0.77	0.04	0.11		0.28
	12	1.25	3.00	0.50	1.37	0.10	0.10		0.33
	40	1.01	2.71	0.41	1.06	0.22	0.08		0.30
	120	0.96	2.66	0.45	1.37	0.17	0.08		0.34
	(360) ¹	0.56	2.90	0.41	1.29	0.20	0.06		0.31)
Mg	0.05	0.20	2.87	0.64	1.12	0.33	0.06		0.69
	0.15	0.50	2.30	0.49	1.06	0.20	0.05		0.46
	1.5	0.80	2.48	0.49	1.26	0.14	0.08		0.24
	15	1.01	2.71	0.41	1.06	0.22	0.08		0.30
	45	0.85	2.68	0.45	1.09	0.12	0.08		0.36
S	0	0.34	2.13	0.72	1.35	0.24	0.08	0.07	0.29
	2.0	0.81	2.51	0.42	1.03	0.12	0.07	0.25	0.26
	6.0	0.84	2.62	0.27	1.10	0.12	0.07	0.24	0.30
	20	1.01	2.71	0.41	1.06	0.22	0.08	0.32	0.30
Fe ¹	0	0.60	2.58	0.50	1.21	0.14	0.07		0.02
	0.02	1.01	2.59	0.53	1.53	0.17	0.08		0.14
	0.2	1.00	2.62	0.45	1.37	0.15	0.07		0.14
	1.0	1.01	2.71	0.41	1.06	0.22	0.08		0.30
± %		10.2	7.3	8.2	11.1	10.7	17.4	(1.4)	36.8

¹ No duplicates.

are less specific than those of birch because they are all associated with chlorosis. Table 7 p. 580 shows certain approximate levels of element content in the needles that correspond to deficiency symptoms. In some cases (N, Ca, S, Fe) the symptoms at one level are so weak as to be described as initial.

Table 4. *Growth values, root/shoot and root weight/root length ratios in relation to supply and content in the needles of the element in question. Control figures in italic type. The dry weights of needles and roots are given in Tables 3 A and 3 B.*

Element	Supply, ppm	Element content of needles, % of dry weight	Total fresh weight, g/beaker	Total dry weight, g/beaker	Stem length, mm per seedling	Root/shoot dry weight	mg dry weight/cm roots
N	0.5	0.7	4.1	1.01	57	0.73	18
	5.0	1.0	12.7	2.77	110	0.60	33
	15	1.7	19.0	4.17	170	0.32	44
	50	2.1	19.1	4.94	182	0.26	78
	150	2.3	19.6	4.50	183	0.26	46
	300	2.4	18.4	4.17	180	0.23	40
P	0.1	0.05	4.2	1.08	61	0.66	23
	1.0	0.11	17.8	4.70	162	0.31	50
	3.0	0.24	19.1	4.87	180	0.27	48
	10.0	0.23	19.1	4.94	182	0.26	78
	30	0.25	20.9	4.50	178	0.27	36
	60	0.32	19.6	4.17	191	0.24	46
K	0.15	0.26	2.3	0.69	68	0.20	14
	1.5	0.31	12.0	3.17	125	0.33	42
	15	0.74	18.1	4.64	191	0.26	67
	50	0.93	19.1	4.94	182	0.26	78
	150	1.09	16.9	4.42	178	0.28	46
	300	1.34	18.1	4.04	190	0.23	38
Ca	0.12	0.02	8.8	2.54	115	0.25	57
	1.2	0.02	13.8	3.80	142	0.25	65
	12	0.12	17.1	4.71	164	0.36	68
	40	0.19	19.1	4.94	182	0.26	78
	120	0.50	19.2	4.70	188	0.26	42
	(360 ¹)	0.99	13.0	3.14	158	0.22	24)
Mg	0.05	0.02	3.9	1.20	88	0.20	17
	0.15	0.02	9.7	2.56	132	0.24	35
	1.5	0.07	10.1	3.46	171	0.30	38
	15	0.11	19.1	4.94	182	0.26	78
	45	0.21	16.4	4.04	169	0.27	41
S	0	0.11	5.6	1.26	102	0.37	19
	2.0	0.13	17.1	3.53	173	0.30	43
	6.0	0.16	17.2	4.14	175	0.25	48
	20	0.18	19.1	4.94	182	0.26	78
Fe ¹	0	0.002	9.8	1.67	107	0.47	37
	0.02	0.003	19.3	4.72	169	0.28	42
	0.2	0.006	19.9	5.02	199	0.27	56
	1.0	0.007	19.1	4.94	182	0.26	78
± e %			6.2	7.4	6.2		

¹ No duplicates.

The symptoms in other cases (P, K, Mg) show at one level but fail to appear at the following higher. In the latter cases the content corresponding to each of these levels is shown in Table 7 together with the relative growth.

Table 5. *Relative total dry weight at varying ion strength but constant proportions of the elements in the nutrient solutions. The control dry weight is put equal to 100 and its element concentrations to 1.*

Concentration	1/2	1/4	1	2	4
Dry weight	68	74	100	85	76

The deficiency symptoms in needles and roots are described in Table 6. The agreement with earlier described deficiency symptoms of spruce is great (cf. 5, 20, 21, 61, 67, 70). Certain discrepancies, however, are recorded. Jessen (20) reports that anthocyanin production was not noticed at phosphorus deficiency in spruce but in the case of pine. In this investigation such a colouration is found in old needles. Furthermore, the chlorosis observed in this experiment at phosphorus deficiency has not been recorded for the seedlings described by Jessen or others. The chlorosis, however, has occurred only in young needles developed at a late stage. Since it is to be expected that terminal buds generally will develop early at deficiency under field conditions, it seems probable that the chlorosis recorded here is rare in the field. The potassium deficiency symptoms are similar to those noted in young seedlings (5, 21, 61). In contrast, mature trees exhibit another symptom with chlorosis in old needles (65, 67).

Characteristic changes of structure occur in the roots at deficiency of cer-

Table 6. *Deficiency symptoms in needles and roots.*

Element	Needle symptoms	Root symptoms.
N	Young needles are bright yellow, especially in the tips. Top buds are occasionally developed.	Very long, thin roots.
P	Young needles are short and yellow, especially in the tips. Old needles are dark green, often with a violet colouring. Top buds are occasionally developed.	Long and thin roots.
K	Young needles are bright yellow but often green at the base and brown in the tips. Top buds are often developed.	Very short, thin and very poorly branched roots.
Ca	Young needles are yellow. All the needles are yellow or brown in the tips. No buds are developed.	Very short roots with many branches.
Mg	All needles are bright yellow with green bases and often with brown tips especially at strong deficiency. No top buds are developed.	Poorly branched roots.
S	The youngest needles are yellow. No top buds are developed.	Thick and poorly branched roots.
Fe	Young needles are bright yellow. No top buds are developed.	Very thick, long roots.

tain elements (Table 6). This is most obvious at calcium deficiency when the die-back of the root tips causes short and densely branched root systems.

The type of root system and the relationship between the root and shoot of young seedlings have been considered of great importance for the development of the seedlings, especially after planting in the field (1, 6, 7). Similar to that of other species the root/shoot ratio of spruce varies at different nutrient conditions (Aldrich-Blake, 1). Yet, Björkman (7) has indicated that the importance of a high root/shoot ratio has been exaggerated, stating it more important that the seedlings have a root system able to develop rapidly in the new site. A root system of this kind is characterized mainly by dense branching and it may be obtained by transplanting in the nursery or by root pruning (7). This statement seems to be well founded since a relatively large but long and thin root system (developed, *e.g.*, under nitrogen deficiency) is heavily reduced at the lifting and planting in order to be manageable from the point of planting technique. The remaining part of the root system may in such a case become rather small in relation to the top.

This investigation presents data that may be used to describe the development of the root system at various nutrient conditions. The type of root system may be expressed by means of dry weight and length. Save for the longest roots, thus avoiding occasional variations, the length of the root system was measured at the harvest. The weight divided by the length of the roots gives an expression of the density and degree of branching. The higher the ratio, the denser and more branched is the root system. Since the diameter of individual roots is varying at acute deficiency, the estimate must be considered to give a ratio that is too high when the roots are heavy (S- and Fe-deficiency), and too low when the roots are slender (N-, P-, and K-deficiency).

Table 4 p. 576 shows the value of root/shoot and root weight/root length ratios at various rates of nutrient application. It is clear that the development and type of the roots to a large extent are affected by the nutrient conditions of the seedlings. High values of the root/shoot ratio are recorded especially at nitrogen and phosphorus deficiency (Aldrich-Blake, 1). It is also evident that the type of root system appears to be disadvantageous at deficiency of these elements since the values of the weight/length ratio are very low. Largely, this depends on the length of the roots (approximately 24 cm at strong nitrogen deficiency and 19 cm at phosphorus deficiency as against approximately 13 cm in the control). The weight/length ratio is highest in the control in all nutrient series and mostly has a very pronounced maximum here. It is possible that this factor may be at least a partial explanation why particularly seedlings produced after optimum and balanced fertilization develop well in forest sites (6, 7, 34, 44).

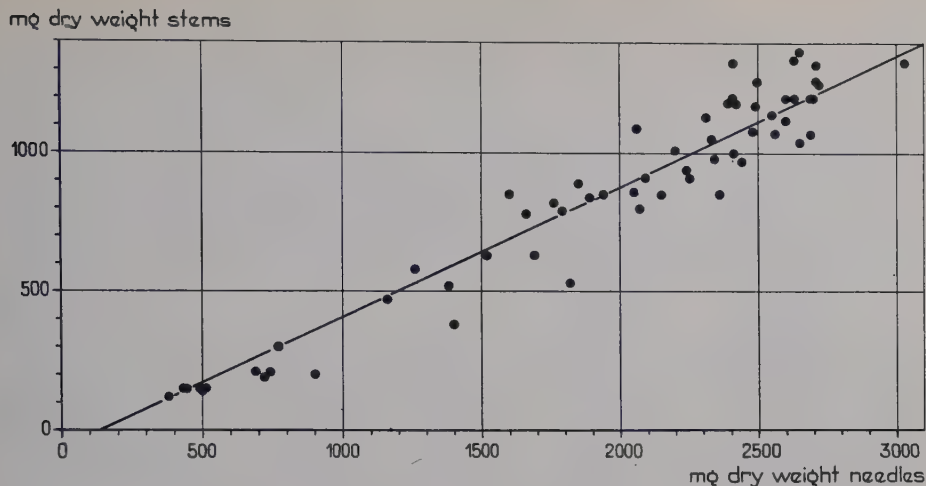


Figure 1. *The correlation between leaf and stem dry weight production within all experiments. The regression is $y = -62.2 + 0.47x$.*

The experiments with birch showed the dry weight of stems and leaves to be very strongly correlated and the ratio between them very closely equalled 0.56 independent of the nutrient factor. Such a correlation exists also for spruce (Figure 1). Each point in the graph corresponds to a beaker value. The correlation is very strong (0.92) and the ratio very close to 0.47, also in this case almost independent of the nutrient status. However, a tendency to declining ratios occurs at very severe deficiency. This may depend on the arrested growth of terminal shoots and needles at some of the lowest rates of nutrient supply. The strong correlation confirms the earlier result (18) that the factors of moisture and light in these experiments are not inhibiting the stem growth at large needle volume as is the case under field conditions (8, 12, 54).

3. Nutrient Absorption and Growth

Analysis and growth data have been compiled in Tables 3 A and B, and 4. A summary of certain critical levels of content is presented in Table 7 for some characteristic physiological conditions of special interest. In Tables 8 and 9, p. 582 f., some of the data are compared with values presented earlier in the literature.

The values compiled in Table 7 are only approximate. Since deficiency symptoms develop gradually, a special threshold value for beginning deficiency symptoms may be difficult to establish. As the deficiency symptoms generally develop within a range where the curve of content/growth is steep,

Table 7. Approximate element content of the needles interpolated from different points on the content/growth curves.

Element	With deficiency symptoms			Element content of needles % of dry weight		
	Symptom strength	Element content of needles, % of dry weight	Growth, % of maximum growth	At 50 % of maximum growth	At maximum growth	At 90 % of maximum growth
N	Beginning	1.0	59	0.9	2.0	1.5 —2.3
P	Strong	0.05	20	0.06	0.2	0.11 —0.3
	No	0.11	90			
K	Strong	0.26	15	0.26	0.9	0.5 —1.3
	Tendencies	0.31	67			
Ca	Beginning	0.02	< 77	0.02	0.08—0.19	0.03 —0.55
Mg	Strong	0.02	50	0.02	0.11	0.09 —0.17
	No	0.07	70			
S	Beginning	0.11	(26	0.12	0.2	0.17 —)
Fe	Beginning	0.003	94	0.002	0.006	0.003—

the content presented in the cases where the deficiency symptoms are judged initial, still must be considered determined with a relatively good accuracy. The relative growth corresponding to symptoms has been graphically interpolated like the content levels that correspond to maximum growth and to 90 and 50 per cent of maximum growth. The values have been computed in a way that differs slightly from that applied for birch (18). In a graph where the relative element content of the needles (per cent of the optimum value) is abscissa and the relative total dry weight (per cent of the maximum value) is ordinate, data from each beaker of an experiment series have been dotted. A curve has been drawn and the maximum found on the curve is judged as *maximum growth* and corresponding element content of the needles as *optimum content*. The maximum growth has subsequently been used as a basis of computation for remaining relative growth values and corresponding levels of content are read from the graph. The content corresponding to 50 per cent of the maximum growth thus has been obtained, as well as the *optimum range* defined by the levels of content that correspond to ≥ 90 per cent of the maximum growth.

As a rule, the dispersion of the point scatter is relatively narrow within the suboptimum range, but increases as the content increases and becomes relatively wide within the supra-optimum range. The values that occur in the suboptimum range, however, attract the greatest interest and are also most accurately determined.

The data provide important information on the needs of spruce for various elements; first, the *absolute requirements* i.e. the amount of element to be absorbed to reach the optimum element content of the needles, and; second,

the *tolerance* to deviations from this content without reducing the growth by more than 10 or 50 per cent of the maximum growth. Since the content corresponding to 50 per cent of the maximum growth is the one most accurately determined, it is particularly feasible for a judgement of the spruce seedling tolerance to the variation of element content. Since no supra-optimum dosages of sulphur and iron have been applied, it is impossible in these cases to interpolate an optimum with satisfactory accuracy. The values presented for these elements, therefore, are course estimates on the basis of the growth curves.

The fact that the measurements have been carried out at the end of the growth period is a source of error affecting the evaluation of the growth and analysis data. The growth must be considered a function of the element concentration during the whole period. It is possible that certain variations in the nutrient absorption have been of importance and that, *e.g.*, the optimum content has been varying. New experiments, however, have been carried out where seedlings of birch, spruce and pine were harvested at various intervals. Data from these experiments will be published shortly. However, certain measures have been taken to avoid occasional fluctuations of the results. Thus, the seedlings have been harvested between two renewals of nutrient solutions and the experiments have been terminated during a period when the seedlings grow rapidly.

4. Nitrogen

The nitrogen content/growth curve is steep on both sides of the optimum which means that the tolerance of spruce to variations in the nitrogen content of the needles is relatively low. Beginning deficiency symptoms occur at approximately one per cent nitrogen in the needles which also corresponds to about half the maximum growth (Table 7). Thus, deficiency symptoms in this case indicate heavily reduced growth. Comparing the content at deficiency with values earlier presented (Leyton, 25, Oksbjerg, 47, Tamm, 64) it is evident that the accordance is very good (Table 8).

The optimum range corresponds to a nitrogen content in the needles of approximately 1.5—2.3 per cent with a growth maximum at 2.0 per cent (Table 7). These values agree well with that assumed by Tamm (64) to be the optimum content for spruce and with the results reported by Mitscherlich and Wittich (33) (Table 9). In other cases where the levels of content corresponding to optimum or near optimum have been suggested, the results seem to be insufficiently supported. Süchting *et al.* (55, 56, 59) reported a nitrogen content in one-year old spruce shoots of 1.2—1.5 per cent to be "normal". The results, however, are obtained in soil cultures fertilized only in the

Table 8. *Some levels of element content corresponding to deficiency reported in the literature. Per cent of dry weight.*

Plant species	Reported by	N	P	K	Ca	Mg	S	Fe
Picea	In the present paper	1.0	0.05—0.11	0.30	0.02	0.02—0.07	0.11	0.003
	Süchting et al. (55—60)	< 1.2	< 0.09	< 0.33				
	Tamm (64)	0.8—1.3	0.07—0.08	0.15—0.30				
	Björkman (5)			0.32				
Pinus	Mitchell (31)	0.7—1.3	0.10—0.28	0.82—1.02	0.23—0.24	< 0.15		
	Tamm (64)	1.1—1.6	0.09—0.10					
	Heiberg & White (16)			< 0.30				
	Stone (52)							
Betula	Ingestad (18)	2.0	0.12	0.3—0.8	0.12	0.06—0.18	0.12	0.003—0.008
	Tamm (64)	1.8—2.2	0.08—0.10	0.29—0.34				

beginning of the experiment and, therefore, nitrogen deficiency has probably occurred at the end of the growing period. Nemec (38, 39, 40, 43) has arrived at very divergent "normal" nitrogen levels in needles. At least in some cases this seems to depend on the fact that the diagnosis has been made on the basis of comparisons between diseased and healthy trees where soil analysis has been used as the main criterion of the nutrient status. Several stands with a nitrogen content in the needles of less than one per cent have been reported (40, 43) without nitrogen deficiency being considered of great importance. In some investigations by Süchting and Nemec also interference with the calcium factor seems to have led to erroneous interpretations. Thus, analysis data often show that the nitrogen and phosphorus content of the needles has increased after liming. The importance of these changes seems to have been underestimated, however.

In some experiments with soil fumigation in forest nurseries (Ingestad and Molin, in preparation) the results indicate that a supply of ammonium sulphate may give a considerable growth increase even when the nitrogen status of spruce seedlings seems to be optimum or supra-optimum. This illustrates that the physiological optimum of a nutrient factor not always is identical with the ecological one and further on that it may be difficult to demonstrate a physiological optimum in a field or soil culture experiment.

The results appear to indicate that the physiological nitrogen requirements of spruce are higher than those previously assumed (cf. Mitscherlich and Wirtlich, 33). The absolute requirements seem to be considerably smaller than that of birch and pine (Table 9). The tolerance of spruce to variations of the nitrogen content, however, seems to be but slightly higher than that of birch.

Table 9. *Some levels of element content corresponding to maximum growth reported in the literature. Per cent of dry weight.*

Plant species	Reported by	N	P	K	Ca	Mg	S	Fe
Picea	In the present paper	2.0	0.2	0.9	0.08--0.19	0.11	(0.2)	(0.006)
	Süchting et al. (55--60)	1.2--1.5	0.16--0.38	0.45--1.25	0.32--1.33			
	Leyton (24)	1.4--1.5	0.13	(1)				
	Tamm (64)	> 2						
Pinus	Mitchell (31)	3.26	0.67	1.72	0.33			
	Tamm (64)	2 --2.5						
Betula	Ingestad (18)	3.6	0.39	(2.9)	0.30	0.34	(0.2)	(0.014)
	Tamm (64)	> 3.2						

5. Phosphorus

Strong deficiency symptoms occur at a phosphorus content of the needles of approximately 0.05 per cent. At 0.11 per cent there are no visible symptoms (Table 7, p. 580). For this reason it is not possible to present any exact element content corresponding to initial deficiency symptoms on the basis of this material. Tamm (64) has suggested values for spruce between 0.06 and 0.08 per cent corresponding to deficiency symptoms. Süchting *et al.* (55, 56, 57, 59) report deficiency when the content is less than 0.09 per cent in one-year old spruce shoots. Nemec (34, 36, 40) has arrived at a similar result and reports a phosphorus content in needles of 0.07--0.13 per cent in spruce at phosphorus deficiency. Everything considered, the results agree very well with the values attained in this investigation (Table 8).

The optimum range of phosphorus is rather wide and corresponds to a content of between 0.11 and 0.3 per cent with a maximum at approximately 0.2 per cent (Table 7). Leyton (24) reports an optimum content of 0.13 per cent of Sitka spruce (Table 9). However, the experiment not comprising definitely established supra-optimal contents, the value may be too low. Süchting (56) found that phosphorus fertilization produced growth increase when the phosphorus content was 0.13 per cent in one-year old spruce shoots, reporting (59) that no deficiency is manifested when the content is 0.16--0.38 per cent. Nemec (34) observed growth response after basic slag fertilization even at a phosphorus content of 0.35 per cent in the needles. The results of his experiments seem to show (table, p. 699) that a growth response appears to be associated with an increase of phosphorus content only when the percentage in the needles is as low as 0.14 per cent. The optimum phosphorus content in this case, therefore, seems to fall within the range 0.14--0.20 per

cent. The growth response recorded despite of the high phosphorus content may depend on secondary effects of the basic slag.

The results seem to indicate that spruce has a relatively great tolerance to changes in phosphorus content in the needles within the suboptimum range. When the phosphorus content is less than 0.10–0.12 per cent, the growth declines rapidly. The tolerance appears to be surprisingly limited within the supra-optimum range but the dispersion of the values here is great. The absolute amount of phosphorus necessary for maximum growth is approximately one-tenth of the nitrogen requirements which agrees well with values reported earlier (Leyton, 24). However, Leyton is basing his statement on probably too low optimum levels of phosphorus as well as nitrogen.

The phosphorus requirements of spruce for maximum growth seems to be lower than those of birch and pine (Table 9). However, the difference between spruce and birch in phosphorus requirements to affectuate half the maximum growth is not so great.

6. Potassium and Calcium

The deficiency symptoms recorded in the potassium series are strong at the lowest rate of application and occurs as trends only at the second lowest rate. Therefore, it seems possible to state that initial deficiency symptoms in this experiment should correspond to 0.3 per cent potassium in the needles. This value agrees closely with those presented earlier (Björkman, 5, Süchting, 55, 56, Tamm, 64). Heiberg and White (16) have reported potassium deficiency symptoms in spruce at a content of 0.13–0.21 per cent without stating any threshold value for beginning symptoms.

The optimum range of potassium content in the needles extends between approximately 0.5 and 1.3 per cent and the potassium content/growth curve has a maximum at approximately 0.9 per cent (Table 7, p. 580). Very few data regarding optimum potassium content in the needles are available. Nemec *et al.* (37, 45) have studied the effect of fertilization with potassium salts but experienced strong contamination with damages because of chlorine ions. Süchting *et al.* (59) report no potassium deficiency in one-year old spruce shoots at a potassium content of 0.45–1.25 per cent which largely concurs with the optimum range found in this study. Leyton (24) reports that the ratio between nitrogen and potassium should be approximately 1.4 at maximum growth which means an optimum potassium content of approximately 1.0 per cent (Table 9, p. 583). Leyton, however, assumes an optimum nitrogen content of 1.4–1.5 per cent which appears to be low. The ratio between nitrogen and potassium is approximately 2 at maximum growth in this investigation.

Initial calcium deficiency symptoms correspond to a calcium content in the needles of 0.02 per cent. As no definitely stated calcium content corresponding to deficiency has been reported, to the author's knowledge, no comparable data can be quoted. The optimum range is very wide (approximately 0.03—0.55 per cent calcium in the needles) and an optimum point is not possible to localize. The calcium content/growth curve is practically horizontal between 0.08 and 0.19 per cent calcium in the needles and the optimum may be established between these values (Table 7, p. 580). Thus, the tolerance to variations in the calcium content is extremely great. The seedlings in one replicate, however, have succumbed at the highest rate of application, but the calcium content in the needles of remaining seedlings in the other replicate is 0.99 per cent which is approximately five times higher than that of the upper optimum limit. Nemec (40, 43) has suggested calcium deficiency in a number diseased stands. The observations are mainly based on soil analysis and on comparisons between diseased and healthy stands. Nemec reports content in the needles down to 0.17 per cent. Süchting *et al.* (59) report 0.32—1.33 per cent as a calcium content "normal" in one-year old shoots.

It seems difficult to compare the calcium content found here with values reported from field experiments. A physiological calcium optimum may differ considerably from an ecological one because of, *e.g.*, the secondary effects of lime in the soil. Moreover, it is possible that calcium in the course of time is largely tied to organic acids of old trees (Chandler, 10). Under field conditions this may mean a toxic effect of calcium at considerably higher content than that found in this study. Since the calcium content of the needles increases with the age (Tamm, 63) comparisons are further impaired. The sampling is not always restricted to special ages of needles (*e.g.*, in Nemec's works). It is likely that the toxicity of the calcium ions is not reflected in the total calcium content of the needles but depends on the amount of soluble and ion-active calcium. The threshold value presented for supra-optimum calcium content (0.55 per cent) consequently appears unfit for a judgement of the calcium status under field conditions.

Björkman (5) and Tamm (61) have discussed the importance of the relationship between potassium and calcium content of soil and needles. Tamm reports that a K/Ca-ratio of 0.5 or less in the needles is associated with potassium deficiency and that a ratio of 1 or more indicates that potassium deficiency is not at hand. The K/Ca-ratio in this experiment is 0.9 when the potassium deficiency symptoms are strong and 1.4 when only signs of symptoms occur. A ratio of 4.9 is recorded at maximum growth and approximately 1 at calcium toxicity. Since a low ratio between potassium and calcium is an indication of both potassium deficiency and calcium toxicity, the ratio appears unsuitable as a measure of the potassium status. The potassium con-

tent itself seems to be more useful for this purpose. In the cases reported by Tamm there are instances of diseased seedlings with 0.72 per cent potassium and 1.36 per cent calcium in the needles which means a K/Ca-ratio of approximately 0.5, *i.e.* within the assumed range of potassium deficiency. Comparably healthy seedlings have 1.11 per cent potassium and 1.41 per cent calcium of the needles, thus a K/Ca-ratio of approximately 0.8. Unfortunately, the needles have not been sufficiently analyzed to exclude the possibility of other deficiencies or toxic effects. However, it is possible that calcium toxicity occurs in this case due to disbalance between the potassium and calcium content in the needles of the diseased seedlings. It has been emphasized that a comparison between the calcium contents in this investigation and such reported from field studies is not reliable. It is likely that the critical K/Ca-ratio (0.5) discussed by Tamm is better suited to field conditions both at potassium deficiency and calcium toxicity than the ratio (1.0) found in this investigation.

The spruce seems to be relatively tolerant to variations of the potassium content and very tolerant to variations of the calcium content of the needles (Table 7, p. 580). The absolute potassium requirements at maximum growth are rather great (approx. half of the nitrogen requirements) and those of calcium very small (approx. 1/20 of the nitrogen requirements). The K/Ca-ratio may provide certain information on a disbalance between potassium and calcium absorption but it seems more advantageous to diagnose potassium deficiency directly on the basis of the potassium content than on the relationship to the calcium content.

Compared with birch and pine, spruce seems to have essentially lower potassium and calcium requirements at maximum growth. At deficiency, however, the values of potassium content are equivalent in all these species. Yet, spruce seems to have lower calcium content at calcium deficiency (Table 8, p. 582) but 50 per cent growth reduction occurs for spruce and birch at approximately equal levels of content.

7. Magnesium

Symptoms of magnesium deficiency are strong at 0.02 per cent magnesium in the needles but fail to appear at 0.07 per cent. Thus, it is not possible to present a value of content that corresponds to beginning deficiency symptoms (Table 7, p. 580). The magnesium content/growth curve indicates that the tolerance of spruce to variations of the magnesium content of the needles is astonishingly limited in the optimum range. In spite of this the magnesium requirements of spruce has attracted little interest, judged from the lack of literature. However, it is possible that the magnesium requirements generally

are satisfied since the absolute requirements are very low and approximately 1/20 of the nitrogen requirements at maximum growth in this experiment.

The optimum range is relatively narrow, especially on the suboptimum side, and is located between approximately 0.09 and 0.17 per cent with the optimum magnesium content of the needles at approx. 0.11 per cent (Table 7). The content/growth curve, however, has an S-shaped course within the suboptimum range. At reduced magnesium content of the needles, therefore, the growth first declines rapidly from maximum, then steadily slower till it drops sharply at approximately 0.02 per cent. Due to this course, the growth reduction amounts to 50 per cent no sooner than the magnesium content of the needles is down at 0.02 per cent despite the narrow range of optimum.

The magnesium requirements seem to be considerably lower than those of birch both at maximum growth and at deficiency (Tables 8 and 9, p. 582 f.).

8. Sulphur and Iron

To the author's knowledge, no works have been published where the analysis of spruce needles with respect to sulphur or iron have been carried out with the purpose of diagnosis. Thus, there are no possibilities to judge the validity of the results found with respect to field conditions. The data presented in this investigation are incomplete since no supra-optimum dosages have been applied. Moreover, at one occasion half of the experiment with iron was supplied with a faulty nutrient solution and but one replicate is reported. Optimum is very indefinitely determined in the sulphur experiments since the sulphur content/growth curve has failed to bend enough to make an interpolation possible. However, new experiments with sulphur and iron will be carried out. Yet, values of content corresponding to beginning deficiency symptoms (Table 7, p. 580) may be estimated with a rather great accuracy, for symptoms started to develop in both series. The values of content corresponding to initial symptoms are 0.11 per cent for sulphur and 0.003 per cent for iron.

Since the iron content/growth curve is horizontal between the control and the second lowest rate of application the optimum iron content presented may be considered relatively dependable (Table 7). For this reason also other critical data are applicable as estimates.

Data available seem to indicate that the sulphur requirements of spruce are approximately equal to those of birch at deficiency level and at maximum growth (Tables 8 and 9, p. 582 f.). The iron requirements of spruce, however, seem to be lower at maximum growth (Table 9) but the differences are slight or non-existent at 50 per cent growth reduction.

9. *Interaction between the Elements*

Increased concentration of an element in the nutrient solution has not only increased the absorption of this element but also enacted changes in the content of other elements in needles and roots. Ion antagonism between potassium, calcium, and magnesium (cf. Burström, 9, Lundegårdh, 27) is generally clearly distinguishable when considering both needles and roots. Since nitrogen has been added as ammonium nitrate, it is possible that the effect of increased nitrogen supply on the calcium absorption may depend on the ion antagonism of NH_4^+ . The potassium content, however, is increasing rather than decreasing at increasing rate of nitrogen application.

An increase of the content of other elements may generally be recorded at deficiency of phosphorus, potassium and sulphur. This may depend on growth reduction without corresponding decrease in the ion absorption. One exception, however, is the fact that the nitrogen content declines at reduced sulphur application, which is evident in the roots. Observed also in birch (18) and other plants (13, 22), this has been considered a consequence of decreased nitrate reduction at deficiency of sulphur and associated reduction of Kjeldahl nitrogen.

10. *Relationships between the Levels of Content in Needles and Roots*

Mostly, the various contents of element in needles and roots are about equal (Tables 3 A and 3 B, p. 574 f.). The content of the roots is often slightly higher than that of the needles. Thus, the elements absorbed seem to be distributed rather uniformly throughout the seedlings. Iron is a distinct exception, since it is not transferred into the needles in proportion to the amount absorbed. Thus, the iron content of the roots is approximately 40 times that of the needles at a balanced nutrient supply. At deficiency of potassium and magnesium the iron content of the roots is approximately 80—140 times higher than that of the needles. At iron deficiency, however, the iron is more evenly distributed.

It is remarkable that proportionately larger amounts of calcium and magnesium remain in the roots at deficiency than at excess dosages of the elements. The element content in the roots is affected to a much lower degree by the supply of these elements than is the content of the needles.

The process is reverse at sulphur deficiency. The sulphur content of the roots is more affected than that of the needles at the lowest rate of application. A high phosphorus, calcium, and magnesium content in the roots may be recorded at sulphur deficiency but is not reflected in a corresponding transfer to the needles.

The magnesium content in the roots decreases at increasing nitrogen supply

whereas it is fairly constant in the needles. The transfer of magnesium increases consequently as nitrogen supply is raised. The magnesium transfer is highest at deficiency in the phosphorus series.

An increase in the transfer of potassium may be recorded at increasing supply of calcium and magnesium within the suboptimum range.

Summary

Spruce seedlings have been grown in nutrient solutions at varying rates of application of N, P, K, Ca, Mg, S, and Fe. Growth measurements and chemical analyses of needles and roots have been carried out. The results may be summarized as follows:

1. Despite low light intensity the growth conditions have been superior to those occurring in the field. The nutrient solution of the control (the common member of all experimental series) has appeared nearly optimum for the elements tested under the conditions prevailing during the experiment.
2. The content in needles corresponding to various physiological nutrient conditions has been approximately determined. The determinations are independable with respect to sulphur and iron since no supra-optimum rates of application of these elements were applied. The absolute requirements of spruce with respect to the other elements (the amount of element needed in the needles at maximum growth) may be elucidated by the following series of declining requirements:

$$N > K > P > Mg > Ca$$

The tolerance to deviations from the optimum content of the needles without a loss of growth exceeding 10 per cent of the maximum varies depending on the element involved. The following series of increasing tolerance may be established:

$$N < Mg < P < K < Ca$$

If a growth reduction of 50 per cent of maximum growth is used as a basis the following series of increasing tolerance is obtained:

$$N < P < K < Ca < Mg$$

The requirements of spruce consequently are highest with respect to nitrogen and the growth reduction will be great if the nitrogen content of the needles is not relatively close to optimum. Calcium constitutes the opposite extreme since spruce has low absolute requirements with reference to this element and the calcium content of the needles may vary within very wide limits without changed growth.

3. The requirements of spruce at maximum growth with respect to various elements are less than those of birch and pine.
4. Deficiency symptoms are relatively non-specific in these experiments and in all cases associated with chlorosis.
5. Dry weight values of stem and needles are very strongly correlated independent of the nutrient status.
6. Development and type of roots are strongly influenced by the nutrient factor. The types of root developed at optimum nutrient conditions seem to be most advantageous with respect to the quality of nursery seedlings.

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Nitrogenous Reserves of Apple Trees

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I. Introduction

Textbooks and handbooks on fruit growing rarely mention nitrogenous reserves as being of importance to fruit trees. An exception is the book "Fundamentals of fruit production" by Gardner *et al.* (1952). Here it is stated "that new tissue growth in early spring is at the expense of stored foods, including stored nitrogen. Hence, for good spring growth of tissues, especially shoots, leaves and spurs, abundant nitrogen storage the previous season is a

prime requisite". In the same book, a table compiled from data made available by Butler *et al.* (1917), shows that the nitrogen of practically all tissues of seven-year-old apple trees is at a minimum when active growth has ceased, and at a maximum at the time of bud swelling. This indicates nitrogen storage. Gardner *et al.* conclude that "the nitrogen that is stored over the winter evidently comes from the leaves".

Many authors treating the subject of the nitrogen nutrition of fruit trees express themselves in terms similar to those of Kobel (1954). He states that no element other than nitrogen leads, upon moderate supplies, so quickly to inhibition of growth, to such an early and marked effect after fertilization, and to such an unpleasant effect when in excess. Statements of this type would suggest that storage nitrogen plays only a small part in the nitrogen metabolism of fruit trees, because the operation of nitrogenous reserves would be expected to act as a buffer against the effects of changes in the external supply of nitrogen.

In textbooks of plant physiology, storage of foods is often treated in connection with translocation, and the importance allotted to storage nitrogen in the life of the trees seems very much dependent on the view taken of the main features of translocation. Miller (1938) finds the indications to be that there is normally no movement upwards to the tips of the branches from the roots, and little or none from the main trunk. Curtis and Clark (1950) conclude that there is probably, under normal conditions, very little upward transport of stored foods from the main branches, trunks or roots. They add, however, "in some perennial plants that do not fruit annually, foods may accumulate in considerable quantity in the trunks and roots during one or more growing seasons. From these storage tissues the foods move up to the developing fruits during years when there is a heavy set of fruit. Under such conditions there may be considerable upward transport".

If the reserve foods are considered alone instead of linked to the problem of translocation, a better starting point for a fair judgement of their value may be obtained. Especially so since the opinions reached by investigators in the field of translocation cannot be said to be very uniform.

According to Thomas *et al.* (1956) a substance should be classed as a reserve food in plants when a period of accumulation is followed by a period in which the substance is maintained at a relatively high concentration, which later diminishes in connection with physiological processes. Thus, no single criterion delineates material as reserve food, though several types of observations would indicate storage of food.

There are a number of observations indicating the storage of nitrogenous reserves in fruit trees. Seasonal variation in total organic nitrogen of several tissues of the apple tree has been shown by the analyses of Butler *et al.*

(1917). Interpreters of these results have placed most stress on the variation in the nitrogen content of the younger branches. This has probably been done under the influence of the observations of Murneek (1930), who found that 35 to 45 per cent of the total nitrogen of the leaves is reabsorbed by the tree prior to abscission. Other investigators have also shown seasonal variation in the nitrogen content of younger branches and shoots of fruit trees (Karmarkar 1934, Thomas 1927).

However, observations have also been made which indicate that the reserves may not solely originate from autumnal migration of nitrogenous compounds from the leaves to younger branches. Nightingale (1934) showed that dormant apple trees absorb nitrogen, and that organic nitrogen compounds are formed at a temperature of 48°F (8.9°C). The experiments were conducted over a 16-day period prior to bud swelling, new roots were formed, and there was no indication of translocation to old roots. Batjer *et al.* (1943) found that appreciable nitrogen, both in the nitrate and the ammonium form, was assimilated by roots of dormant apple trees subjected to temperatures as low as 32 to 33 degrees F (< 1°C). Further they state that in fruit regions with a rather mild winter, where even the surface soil is seldom frozen for any great length of time, lack of nitrogen absorption is probably due to factors other than soil temperature. Aldrich (1931) carried out experiments in which apple trees were fertilized three times during the fall and winter. There was an increase in total nitrogen of the feeding rootlets during the winter. His conclusions are that the roots were taking up nitrogen from the soil, and further that the nitrogen taken up by small rootlets was translocated into the larger roots. Sullivan and Kraybill (1930) found an increase in all forms of nitrogen in all parts of the apple tree between December and February, indicating that both absorption of nitrogen from the soil and synthesis of protein was taking place. The delay of up to four years in any marked effect of a decrease in the amount of nitrogen given to apple trees which previously had been heavily fertilized, was taken by Oland (1955) to indicate storage of nitrogen. Williams (1931), and Weinberger and Cullinan (1934) found that peach trees during the season of dormancy absorbed large amounts of nitrogen applied in the autumn and winter.

Roberts (1921) and Oland (1954) have shown that young apple trees in sand culture may grow appreciably without external supply of nitrogen.

Little seems to be known about the nature of the reserve nitrogen of the apple tree. The storage foods of plants are often stated to be proteins, carbohydrates, fats, and oils (Miller 1938, Thomas *et al.* 1956, Stiles 1950). According to Nightingale (1937), it is beyond doubt that the nitrogenous reserves of many seeds for the main part would be proteins, and in some cases reserve proteins have been isolated (Danielson 1951), but this need not apply to the

reserves of trees. On the contrary, some evidence exists that the nitrogenous reserves of the trees and of storage organs in many plants for the main part may be low molecular weight compounds. Rahn (1932) showed that a large part of the nitrogen in the storage organs of *Allium cepa*, *Oxalis deppei*, and *Asparagus officinalis* is in soluble, low molecular weight compounds. The recent observations by Mothes and Engelbrecht (1952) on the occurrence of allantoic acid and allantoin in *Acer platanoides*, lead to the conclusion that these are the main nitrogenous reserve compounds of that species. Reuter (1957) determined by paper chromatography the "main nitrogenous compounds", soluble in 80 per cent ethanol, of storage organs of 166 species belonging to 48 plant families. By "main compound" was meant the amino acids that constituted most of the amino nitrogen. Among these main compounds should be mentioned arginine and asparagine of the Rosaceae, citrulline of the Betulaceae, acetylornithine of Fumaroideae, and proline of Papilionaceae as well as glutamic acid, glutamine, aspartic acid, asparagine and azetidin-2-carbonic acid in several species. Analyses of all parts of young apple trees showed that plants which had not received nitrogen during the previous growing season were depleted of soluble nitrogen, while large amounts had accumulated in those which had received nitrogen (Oland 1954). Arginine and asparagine were shown to be much more abundant in the soluble fraction of the N-rich than of the N-poor plants. These results emerged only from visual judgement of spots on paper chromatograms.

Apart from experimental evidence indicating the nitrogenous reserves to be low molecular weight compounds, statements implying this fact are found in literature (Steward *et al.* 1954, Oland 1954, Reuter 1957). The statement that the free amino acids may have a main function as nitrogenous reserves, is supported by the fact that they may accumulate in considerable quantities, and by the lack of evidence of any quantitative demand for amino acids even where the metabolic activity is supposed to be vigorous.

In a review on nitrogenous constituents of plants, Steward and Thompson (1950) state that "a challenging need is to describe the normal growth and development of cells, organs, and of whole plants, in terms of an orderly sequence of events interpretable in terms of their nitrogen compounds . . . Though some of the outstanding problems can be simply stated, their analysis in terms of actual data is possible only to a limited extent". This general statement on lack of knowledge, may be said also to embrace the question of the importance of nitrogenous reserves in trees.

The aim of the present investigation was to study the importance of the nitrogenous reserves for young apple trees, to obtain knowledge of the nature of these nitrogenous reserves, and to investigate chemical methods which might be reliable when applied to trees under orchard conditions.

II. Analytical Methods

Plant material for analysis. Fresh, dried, and frozen plant material was used for analyses. When fresh material was taken, it was used immediately after collection from the greenhouse. It was cut finely with a knife and either extracted in a homogenizer (16,000 r.p.m.) or ground to pass a 20-mesh sieve and afterwards extracted as dried material. Material for drying was also cut finely and dried at 70°C to constant weight, usually for at least two days. It was then ground to pass a 40-mesh sieve, and kept at room temperature in bags of grease-proof paper until used for analysis. The plant material for freezing was also cut finely as soon as possible after collection, wrapped in grease-proof paper and frozen at -25°. When needed for analysis, it was ground on a cooled mill in a cold room to pass a 20-mesh sieve. Some samples contained too much moisture to be ground in this way. In such cases the sample was left in an open bag in the freeze-box until sufficient water had evaporated to allow grinding.

When analyses were performed on either fresh or frozen material, samples for determination of dry weight were taken at the same time as were samples for analyses, so as to allow the results to be expressed on the basis of dry plant material.

Total organic nitrogen was determined by the Kjeldahl method, selenium being used as a catalyst. It was found necessary to weigh approximately 0.25 g of plant material for digestion, dilute to 50 ml after this procedure and take a 10 ml aliquot for distillation. In this way a fairly high precision was obtained, whereas the results obtained with the use of about one-fifth of the above-mentioned sample size were less satisfactory. Duplicate determinations were usually performed on every tenth sample. According to the figures thus obtained, the standard error on the single determination has been ± 2.3 per cent.

Total soluble nitrogen. The total nitrogen content of the extracts was determined by the Kjeldahl method. The digested sample was transferred directly from the Kjeldahl flask to the distillation apparatus. The standard error on the single determination has been ± 1 per cent.

Non-soluble nitrogen is the difference between total nitrogen and soluble nitrogen.

Determination of "amino nitrogen" in the crude extracts was carried out according to the method of Yemm and Cocking (1955). Attention was paid to a careful reading of comparable blanks. The value of the method, used in this way, is limited by the fact that the amino nitrogen of compounds other than the α -amino acids does not react to give a 100 per cent colour yield during the 15 minutes heating-time. Therefore, the method was used in a few cases only, for comparison of samples with the same general composition.

Determination of arginine in crude extracts was carried out according to the Macpherson (1942) modification of the Sakaguchi reaction.

Dialysis was performed on some of the extracts. The procedure employed by Danielson (1951) was followed. The facilities for running dialyses at low temperature (+4°C) were, however, unsatisfactory. The dialyses were partly run in the refrigerator with frequent changes of water, and partly in the laboratory, the dialysis chamber being cooled down with ice. The 0.2 M NaOH extracts were neutralised with HCl before being enclosed in the dialysis tube.

Column chromatography of amino acids. At the beginning of this work the original method of Moore and Stein (1951) was employed. The extracts were run on the 15 cm column only, which separates arginine, ammonia, lysine, histidine, γ -amino

Table 1. Recovery from the 15 cm column of some ninhydrin reacting compounds.

Compound	1			2		
	Column load $\mu\text{g N}$	Recovery $\mu\text{g N}$	Recovery per cent	Column load $\mu\text{g N}$	Recovery $\mu\text{g N}$	Recovery per cent
Glutamic acid	14.0	14.78	105.6	28.0	28.94	103.4
Asparagine	56.0	56.35	100.6	168.0	165.76	98.7
β -alanine	14.0	14.36	102.6	28.0	28.44	101.6
γ -amino butyric acid	14.0	13.94	99.6	7.0	7.02	100.3
Histidine	74.0	70.28	95.0	42.0	41.64	99.1
Lysine	28.0	27.86	99.5	56.0	54.48	97.3
Ammonia	14.0	14.75	105.4	28.0	29.08	103.9
Arginine	168.0	168.56	100.3	112.0	112.43	100.4

butyric acid, and β -alanine while the acidic and neutral amino acids emerge in a bulk. The final determination of the eluted compounds was carried out according to the method of Yemm and Cocking (1955). Table 1 shows the recovery of amino acids with this procedure, which was used to obtain the results of figures 3, 4 and 5. Later, a somewhat different procedure was used, kindly suggested privately by Drs. E. W. Yemm and A. P. Sims. The main alteration consisted in changing the 8 per cent cross-linked sulfonated polystyrene resin to a 12 per cent cross-linked one of the same manufacture ("Zerolite"). This ion exchange material has a higher resolution power than the 8 per cent cross-linked. A considerable gain of time was achieved, as fifteen 1 ml fractions could be collected every hour against four per hour in the original procedure. At the same time an analysis was completed in fewer fractions. The outlines of the modified procedure and the elution pattern are given in Figure 1.

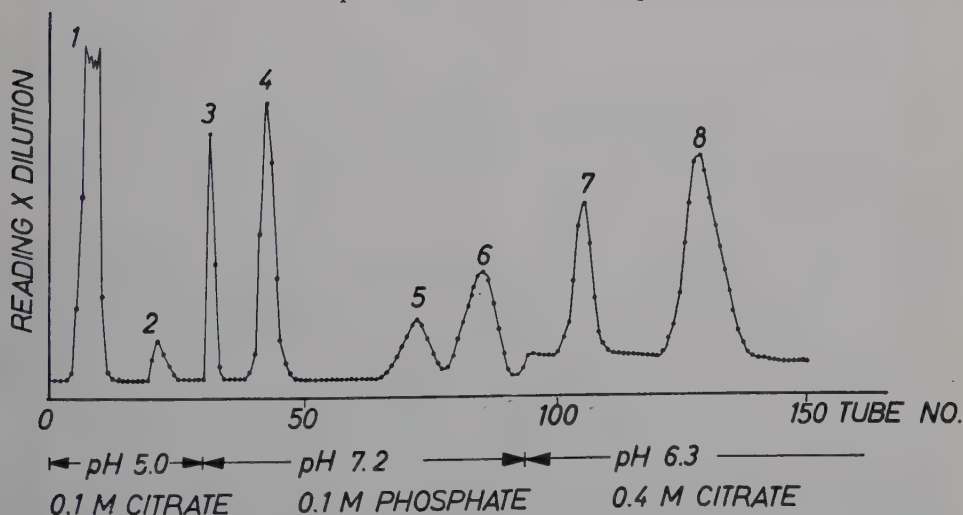


Figure 1. Elution pattern of a 12×1 cm column of 12 per cent crosslinked "Zerolite" cation exchange resin. 1) neutral and acidic amino acids+the amides, 2) phenylalanine, 3) γ -amino butyric acid, 4) histidine, 5) ornithine, 6) lysine, 7) ammonia+ethanolamin, and 8) arginine. Temperature of the column 15°C until tube 15, and later 40°.

1 ml/4 minutes.

The 0.1 *M* citrate buffer of pH 5 is the same as that used by Moore and Stein (1951). The 0.1 *M* phosphate buffer of pH 7 was prepared by mixing 0.1 *M* NaH_2PO_4 and 0.1 *M* Na_2HPO_4 to give the desired pH value. Approximately 0.45 litre of the former was used with a litre of the latter. The 0.4 *M* citrate buffer of pH 6.3 is the 0.2 *M* pH 6.5 buffer of Moore and Stein (1951), only of increased molarity and adjusted to pH 6.3 with concentrated HCl. Versene was added to the buffers as recommended by Moore and Stein (1951).

Quantitative determination of N in amides, and the group of acidic and neutral amino acids. As will be seen from Tables 8 p. 611 and 20 p. 633, a separation between the amides and the neutral and acidic amino acids has been attempted.

For the determination of the amides the method of Varner *et al.* (1953) was investigated. It was found: 1) that 40 per cent NaOH had to be exchanged with saturated NaOH (at room temperature) in order to obtain a quantitative determination of the amide N of asparagine. 2) Pure solutions of arginine yielded large and varying amounts of ammonia when distilled as recommended for the amide determination. 3) Citrulline also yielded small amounts of ammonia. Thus, there may be large constant errors on the determination of amide N when this method is applied to extracts which also contain, for instance, arginine, a quantitatively important amino acid in much plant material.

However, it was found that this simple method for amide determination could be applied, with high precision, to the fractions from the ion exchange column containing the amides and the neutral and acidic amino acids. All the amino acids likely to occur in these fractions, according to Oland (1954), have been tested, and found not to yield ammonia when boiled with saturated NaOH in the Parnass-Wagener apparatus for 5–8 minutes. Citrulline would emerge in these fractions if present in the plant extracts, but is not likely to be found in significant amounts in the apple tree (Oland 1954).

The sum of acidic and neutral amino acids and amides was determined by the ninhydrin method of Yemm and Cocking (1955). In order to obtain 100 per cent formation of DYDA (diketohydrindylidene-diketohydrindamine) also from asparagine, the heating time was prolonged to 50 minutes from 15 minutes which are only sufficient for 45 per cent formation of DYDA. The only drawback of prolonged heating time seems to be high blank readings.

The values in the tables for acidic and neutral amino acids are obtained by subtracting the value for amide N from the value for amino N in the same fractions. Actually, two separate samples have been run on the ion exchange column. The one for determination of amide N has usually contained four times as much nitrogen as the one for complete analysis on the column. This was done in order to obtain a more convenient nitrogen content for accurate volumetric analysis.

Glutamine is probably only found in very small quantities in the roots and stem, but may be more abundant in the maidens (whips) according to Oland (1954). An examination of the behaviour of glutamine may, however, still be required as changes in glutamine are known to occur on ion exchange columns (Steward and Pollard 1957). As is seen from Table 2 glutamine in pure solutions could be recovered quantitatively if the temperature of the column was kept at 15°C, while full recovery was not obtained with a column temperature of 40°. These observations led to the precaution that the column was kept at a temperature of 15° till fraction 12 had emerged. The final determination of glutamine could be done either as amide N or as amino N.

Table 2. *Recovery of glutamine from the 15 cm column at two different temperatures.*

Temperature °C	Column load µg N	Recovery µg N	Recovery per cent
40	56.0	38.51	68.7
15	56.0	54.73	97.7

Protein precipitation was performed on 20 ml aliquots of extracts by adding 1 g of trichloroacetic acid. The mixture was allowed to stand 50 minutes before it was filtered.

III. Extraction of Free Amino Acids

The precision of a method may be judged from experience, or by statistical means as soon as an appropriate set of data is available. Constant errors are usually revealed when two or more quite different experimental procedures are employed to measure the same quantity.

In the author's opinion the search for constant errors should, as often as possible, be included in chemical work with biological material. For the present work it was of special interest to know the merits of the various chemical methods, since information was wanted on the composition of the nitrogenous reserves.

The aim of the extraction was to separate the low molecular from the high molecular weight nitrogen compounds.

Seventy to eighty per cent aqueous ethanol, either cold or hot, is probably the extractant most frequently used to obtain the so-called free amino acids from plant material. But extractants such as hot or cold water, buffers and dilute acids have also been used for an initial fractioning of the nitrogenous compounds. Discrepancies in the quantity of non-proteinaceous nitrogen have occurred when two or more extractants or procedures have been used on the same plant material (Steward and Street 1946, Bisset 1954). This obviously calls for exertion of care in the choice of extractant.

Comparison of different extractants. As an initial step the completeness of the nitrogen extraction by aqueous ethanol was investigated. Figure 2 shows the results of repeated extraction of finely divided apple stem tissue with a 70 per cent aqueous ethanol. The extraction of nitrogenous compounds was slow; comparatively little nitrogen was extracted after 500 ml of extractant had been used.

Table 3 shows the amount of nitrogen extracted with a variety of extractants and extraction procedures. It is noticeable that several extractants and procedures yielded the same amount of soluble nitrogen, and that the major

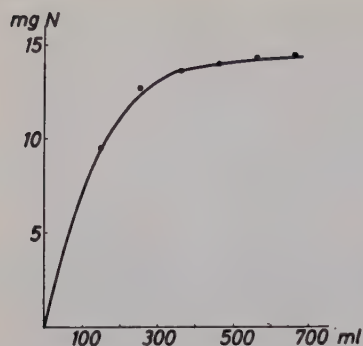


Figure 2. Cumulative curve for the nitrogen, obtained by repeated extraction of 5 grammes of plant material with new lots of 70 per cent aqueous ethanol.

part of this nitrogen dialysed against water through a cellophane membrane. Seventy per cent aqueous ethanol extracts less nitrogen than any other of the tried extractants. Samples thoroughly extracted with 70 per cent ethanol, yielded, upon continued extraction with hot water or 0.2 *N* sodium chloride, a nitrogen amount equal to the difference between the amount extractable from parallel samples by hot water alone and that extractable by ethanol. The 0.2 *N* sodium hydroxide solution, which might be expected to be a severe extractant, dissolved the highest amount of nitrogen from the sample, but quite a high percentage of this nitrogen did not dialyse against water. Dialysable nitrogen extracted with the latter equals closely that extracted with 0.2 *N* sodium chloride.

Table 3. Total soluble *N* and non dialysable *N* with different extractants and different extraction procedures. Each figure (mg *N*/100 g dry matter) is the mean of parallel determinations on three different samples with total organic nitrogen content of 624, 961, and 1141 mg/100 g dry material, respectively

Plant material g	Extractant	Procedure	Total soluble N	Soluble N after precip. w. tri-chloroacetic acid	Non-dialysable N
1	100 ml 0.2 <i>M</i> NaCl pH 7 buffer	24 hrs. at 4°C + shaking	529.5	531.1	43.3 ¹
1	"	percolation	532.7		
1	100 ml dest. water	10 min. at boil. p.	531.9	530.6	42.2 ¹
1	"	24 hrs. at 4°C + shaking	477.5		
2	500 ml 70 % ethanol	repeated extraction with new lots	380.6		
2	100 ml dest. water following the eth.	10 min. at boil. p.	151.3		
1	100 ml 1 <i>M</i> NaCl	24 hrs. at 4°C + shaking	533.9		
1	100 ml 1/100 <i>N</i> HCl	"	531.4		
1	100 ml 1 <i>N</i> HCl	"	530.2		
1	100 ml 0.2 <i>N</i> NaOH	percolation	608.3		117.8 ¹

¹ Mean value of three determinations.

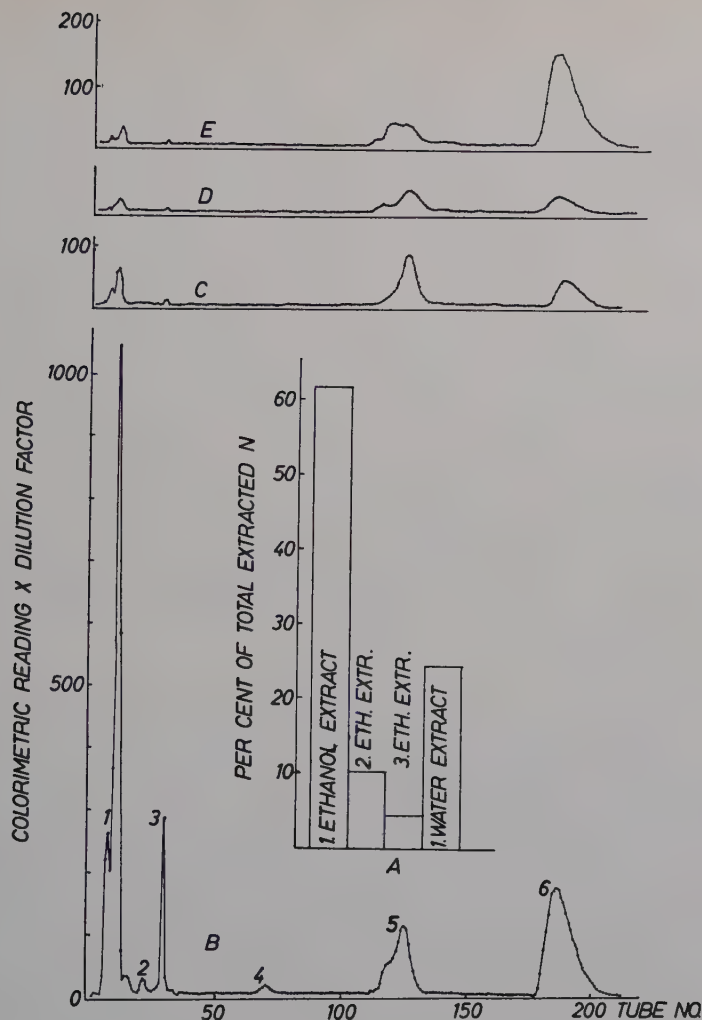


Figure 3. Total extracted N, and the peaks on the 15 cm ion exchange column of three subsequent ethanol extracts, and one water extract of the same plant material. A) graph of total extracted N, B) the peaks of the 1st ethanol extract, C) the 2nd ethanol extract, D) the 3rd ethanol extract, and E) the water extract. 1) acidic and neutral amino acids + the amides, 2) β -alanine, 3) γ -amino butyric acid, 4) histidine, 5) ammonia, and 6) arginine.

It is also shown in Table 3 that nitrogenous compounds are not precipitated by the protein precipitant, trichloroacetic acid, in the extracts obtained by the pH 7 buffer or hot water.

From these results it follows that all the low molecular weight nitrogenous compounds were not extracted with 70 per cent ethanol, although the shape

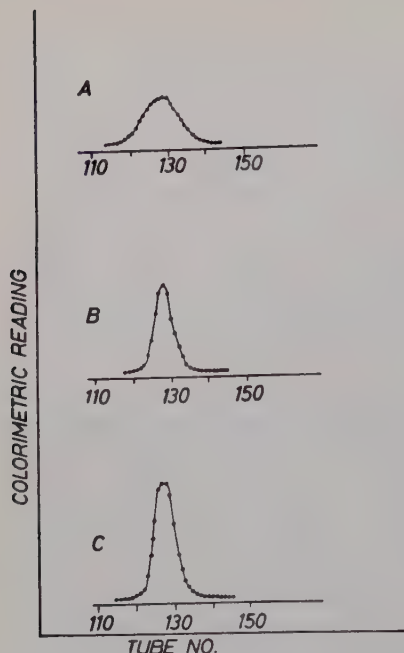


Figure 4. Peaks at tube 126—128 of A) ammonia, B) ethanolamine, and C) ammonia+ethanolamine.

of the extraction curve indicates complete extraction of the compounds which could be removed with that solvent.

Extraction of arginine. If the low molecular nitrogenous compounds, not extractable with 70 per cent ethanol, were amino acids, this should be revealed by analysing consecutive extracts on the ion exchange column. Figure 3 pictures the results of the analyses on the 15 cm column of three consecutive ethanol extracts and one water extract of the same sample of plant material. All the extracts were concentrated in vacuum at a temperature of 40°C before analysis. The graph of total nitrogen in each extract shows the extraction of this sample to conform with earlier extractions with ethanol followed by water. For reasons which will be apparent later, little importance should be attached to the actual quantities, which can be calculated for some of the amino acids represented by a peak in this figure. However, from a rough estimate it is clear that practically all the acidic and neutral amino acids and the amides are removed from the tissue by the first ethanol extraction, while arginine is still present in substantial quantities in the second and third ethanol extracts. In the final water extract, arginine is present in a quantity of the same order of magnitude as in the first ethanol extract.

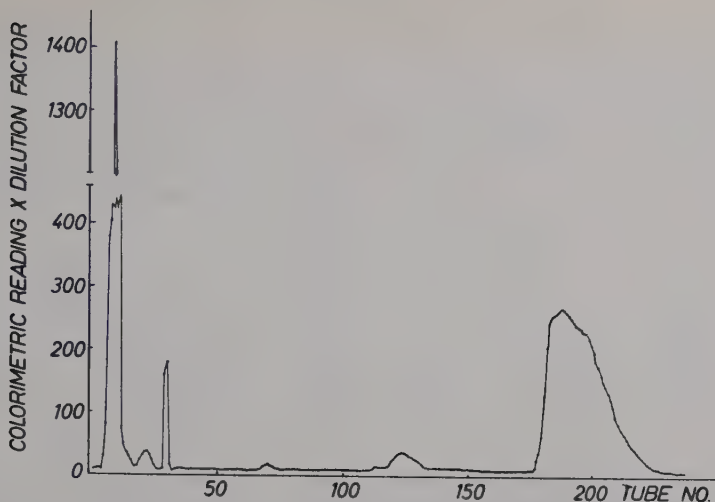


Figure 5. The peaks on the 15 cm column of a pH 4 citrate-buffer extract. Equivalent quantity, and of the same plant material, as used for the analyses of Figure 3. The figure on same scale as Figure 3.

The peak which according to Moore and Stein (1951) should represent ammonia, is at about tube 125. In the ninhydrin procedure of Yemm and Cocking (1955) ammonia reacts to give a colour equivalent to only about one-third of that of pure DYDA (diketohydrindylidene-diketohydrindamine; the purple colour of the reaction is attributed to this compound). Thus, the fairly large peaks at tube 125 in Figure 3 might represent large quantities of nitrogen. Together with the inconsistent shape of the peaks, this was thought a strong indication that the analytical procedure was subject to a serious constant error.

As is shown in Figure 4, not only ammonia but also ethanolamine has a peak around tube 125. From a comparison of the peak shapes of these two compounds, and the shape of the peaks at tube 125 in Figure 3, it seems fair to conclude that ethanolamine may be present in the extracts represented in Figure 3. Some of the peaks at tube 125 have a shape indicating "double-peak". Ethanolamine has been shown earlier to occur in extracts of apple trees, as revealed by paper chromatography (Oland 1954). As to the shape of the ammonia peak, the results of Moore and Stein (1951) and the present results are not in complete agreement. It may be due to ion exchange material from different sources, or to minor differences in buffers or procedure.

In order to make it possible to conclude reliably whether both ammonia and ethanolamine are present in the peak at tube 125, some work was done to find buffers which might separate these two compounds on the short column. The work was, however, unsuccessful.

A parallel sample to that used for the analyses in Figure 3, was extracted with a pH 4 buffer (0.1 M sodium citrate adjusted to pH 4) at a temperature of about 4°C. A small amount of extractant was used, so that no concentra-

Table 4. Arginine content in final hot-water extract of apple "stem" material after previous extraction with 70 per cent ethanol. Sample size ca 2 g on a dry weight basis.

Sample no.	mg total N in ethanol extracts			Final water extract			Total extracted N mg
	1st.	2nd.	3rd.	Total N mg	Arginine N		
					mg	%	
7				7.77	2.45	31.5	7.77
8				7.78	2.18	28.0	7.78
1	5.78			2.24	1.53	68.4	8.02
2	5.90			2.21	1.51	68.3	8.10
3	5.55	0.77		1.47	1.02	69.4	7.79
4	5.78	0.88		1.51	1.16	76.3	8.18
5	5.60	0.94	0.09	1.12	0.93	82.9	7.74
6	5.56	0.89	0.09	1.19	0.92	77.4	7.72

tion was needed before the extract was put on to the column. This extraction was expected to allow but small possibility for changes in the nitrogenous compounds since the handling of the extract was at a minimum. The amount of nitrogen extracted with this small amount of extractant was approximately 90 per cent of the amount extractable with hot water or with the pH 7 buffer according to the procedure of Table 3.

Figure 5 shows the results of the analysis of this extract on the short column. The peak at tube 125 is small in comparison with the peaks at the same position in Figure 3, which represent the same amount of plant material. This can only mean that qualitative changes have taken place in the nitrogenous compounds of the ethanol and hot water extracts during the extraction and subsequent concentration. The changes have presumably led to increased amounts of ammonia or ethanolamine, or both or possibly of some other compounds which may emerge from the column in the same position.

From measurements of the areas of the arginine peaks in Figures 3 and 5, it is apparent that the peak of Figure 5 represents approximately 30 per cent more arginine than the four peaks in Figure 3 together. It is thus indicated that arginine has been lost by the ethanol extraction and the concentration of the extracts.

In order to get some idea of whether arginine is the only compound not fully extracted by 70 per cent ethanol, and thus probably the origin of the compounds appearing at tube 125, the following experiment was conducted. Different portions of the same plant material were extracted either with hot water or with ethanol in one, two or three consecutive processes, followed by a single extraction with hot water. Determinations were made of total N in all extracts and determinations of arginine, by a modification of the Sakaguchi reaction, in all the water extracts. This allowed a calculation of the

Table 5. *Experiments with soaking of nitrogen-poor plant material in three different solvents and in the same solvents containing dissolved glutamic acid and arginine-HCl at two different levels.*

Added to the sample of plant material (20 ml to 2.5 g fresh material)	μ g amino-N determined with the ninhydrin procedure				
	Initial amount	after 30 min	after 4 hours	after 9 hours	after 25 hours
1. 70 per cent ethanol	0.0	42	90	110	122
2. Distilled water	0.0	96	110	120	114
3. pH 7 buffer (Danielsson)	0.0	112	122	132	130
4. Glutamic acid in ethanol	204	236	282	298	314
5. " " " dist. water	203	304	322	320	320
6. " " " pH 7 buffer ...	199	316	326	332	330
7. Arginine in ethanol	201	201	196	192	194
8. " " " dist. water	200	236	238	236	236
9. " " " pH 7 buffer	206	306	326	332	328
10. Glutamic acid in ethanol	609	609	609	622	624
11. " " " dist. water ...	596	684	726	722	728
12. " " " pH 7 buffer ..	604	718	730	732	732
13. Arginine in ethanol	612	482	394	378	364
14. " " " dist. water	606	568	518	518	514
15. " " " pH 7 buffer	603	694	722	726	724

arginine percentage in the last extract. The results are found in Table 4. It shows increasing percentage of arginine in the final hot water extract with increase in the thoroughness of the previous extraction with 70 per cent ethanol. The reverse is thus true for the nitrogen not accounted for in arginine. This would indicate that the ethanol extraction may also be slow for compounds other than arginine. Thus, no certain conclusion should be drawn as to whether arginine is a compound particularly liable to breakdown when extracted by and concentrated in ethanol.

The results of this experiment confirm the results obtained on the ion exchange column, that arginine is difficult to extract by aqueous ethanol, and that large amounts of arginine may occur in the apple tree material.

Interaction between amino acids and extractants. In removing the free amino acids from apple tree tissue, there is obviously an interaction between the amino acids and the extractants. This is made manifest by the difficulty of extracting arginine quantitatively with 70 per cent ethanol and the ease of extracting it with other solvents. In order to elucidate this interaction further, the experiments reported in Table 5 were conducted.

Apple-stem tissue from plants which had been grown the previous year without nitrogen in the nutrient solution was cut and ground fresh to pass a 20-mesh sieve. To 2.5 g portions of this ground tissue, containing approximately 33 per cent of water, were added 20 ml of three different extractants, 70 per cent aqueous ethanol, distilled cold water, and 0.2 M NaCl buffered

with phosphate to pH 7. The same extractants, containing two different concentrations ($0.72 \cdot 10^{-3} M$, and $2.16 \cdot 10^{-3} M$) of glutamic acid and arginine, were also added to separate samples of the same plant material. At four-hour intervals, 0.1 ml of each solution was removed and the "amino nitrogen" determined by the ninhydrin procedure.

The relative extracting power of these three extractants is seen from the three first lines of the table. Seventy per cent ethanol extracts slowly, especially if compared to the very rapid extraction with the buffer.

The presence of glutamic acid has interfered with the ethanol extraction; this is especially marked in the highest concentration. Neither of the concentrations of glutamic acid has reduced the power of either cold water or the buffer to extract amino nitrogen from this nitrogen-poor plant material.

The amount of arginine originally dissolved in the 70 per cent ethanol has decreased with time in the presence of plant material. This is true for both concentrations, and is taken to indicate that arginine was adsorbed to the plant material. The same seems to be the case with arginine in cold water. With the lowest arginine concentration the actual amount of amino nitrogen has increased. However, the increase is not sufficient to account for all the amino nitrogen extractable from the plant material. As this simple analysis tells nothing about the amino acid composition of the analysed solution, it may well be that amino nitrogen was removed from the plant material by the extractant at the same time as the plant material adsorbed arginine from the solution.

The pH 7 buffer extracted practically the same amount of amino nitrogen in the presence of both concentrations of arginine.

The choice of extractant and extraction procedure. For routine determination of total soluble nitrogen 0.2 M NaCl buffered with 0.03 M Na_2HPO_4 and 0.02 M NaH_2PO_4 to pH 7 was chosen. For safety the extraction should be performed at a temperature around 4°C , and the extraction time should be one day. This extraction would yield little non-dialysable nitrogen. If the proportion plant material: extractant is 1 : 100 it also seems safe to conclude that this buffer would extract arginine fairly completely, even from plant material very rich in this compound.

Extraction for subsequent analysis on the ion exchange column. For determination of the amino acid composition of the extract by analysis on the ion exchange columns, the extractant and procedure outlined above would not be desirable, as both concentration and desalting would be needed prior to analysis.

It was anticipated that an extraction which could be performed at low temperature, with the same buffer as that needed for the column, would give rise to as little change as possible in the nitrogenous compounds. Some

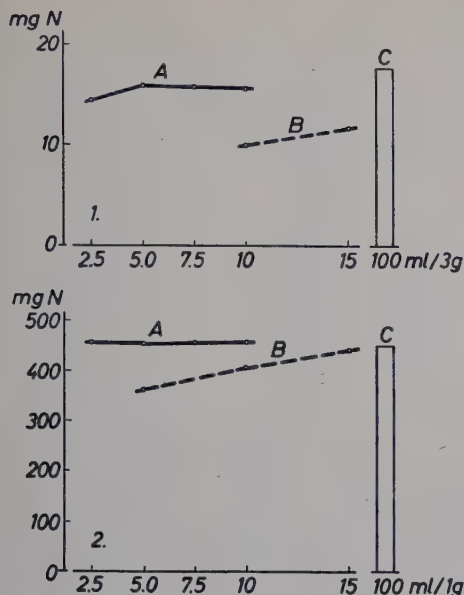


Figure 6. Extraction of stems with small amounts of extractant (pH 4 citrate buffer) for subsequent analysis on the ion exchange column. 1) apple stem poor in nitrogen 2) apple stem rich in nitrogen, A) percolation procedure, B) batch procedure, and C) standard procedure for soluble nitrogen. Mg N extracted, on 100 g dry matter, by 100 ml extractant to 3 or 1 g plant material.

experiments were therefore conducted to obtain the extractable compounds completely with small quantities of buffers.

Figure 6 shows the amount of nitrogen extracted with two different procedures, from comparatively nitrogen-rich and nitrogen-poor plant material. The batch procedure consisted in placing the plant material in buffer and centrifuging after equilibrium had been reached overnight at 4°. In the percolation procedure the ground plant material was packed in an 8 mm glass tube with a narrow tip plugged by glass wool. The extractant was allowed to percolate through the plant material until the desired quantity of extract had been obtained.

As seen from Figure 6, the percolation procedure is superior to the batch procedure in the extraction of nitrogen from the apple stem tissue. The curve for nitrogen extracted by percolation of nitrogen-rich plant material shows that the extraction was complete with the use of 2.5 ml extractant to 1 g plant material on a dry weight basis. A buffer quantity of 15 ml to 1 g plant material in the batch procedure approaches closely complete extraction. The extraction is considered as complete when a nitrogen quantity is obtained equal to that extracted by 100 ml pH 7 buffer.

Three grammes of nitrogen-poor plant material were used, and extracted with the same quantities of buffer as the nitrogen-rich plant material. The lowest quantity of extractant used did not complete the extraction of this amount of plant material, but the three higher quantities removed the same

Table 6. *Total soluble N and soluble dry matter after extraction with pH 7 buffer of fresh, frozen, and dried stems and roots.*

Plant material	Days after harvesting	Total soluble N mg/100 g dry material	Soluble dry matter g/100 g dry material
Fresh stem	0	144.2	9.08
Dried stem	25	144.5	11.51
Frozen stem	25	145.1	9.22
Fresh roots	0	319.1	17.54
Dried roots	25	322.9	24.05
Frozen roots	25	320.7	18.12

amount of nitrogen, and thus all the soluble nitrogen was apparently removed by percolation. The percolation procedure did not extract quite as much nitrogen as the 100 ml pH 7 buffer by the standard procedure. But although significant when measured against the analytical error, the difference would not be of any significance for the choice of procedure in the present studies. This will be apparent after the data on the composition of the soluble nitrogen has been presented, where it is shown that it is not clear what compounds are obtained in the soluble fraction of the nitrogen-poor plants.

The concentration of nitrogen in the percolation extracts of the nitrogen-poor plant material would be sufficient for direct analysis on the ion exchange column. Up to 4 ml of extract may be put on to the column, and an amount of amino nitrogen of 100 μ g would be sufficient for very accurate determinations of many of the separated compounds.

Thus, extraction by percolation with small amounts of buffer would be a solution to the problem of obtaining extracts of this plant material for subsequent analysis on the ion exchange column. The routine procedure has been to pack a glass column with 0.5 to 3.5 g of plant material, according to nitrogen content, and then allowing a 0.1 *M* citrate buffer of pH 4 to percolate through the column until 10 ml of extract were obtained.

Citrate and phosphate buffers of pH from 3 to 8 have been tested for their power to extract nitrogen by percolation. They were all found to be equal.

Extraction of fresh, frozen and dried plant material. Chemical analyses of plant material are usually very time-consuming. The number of analyses which can be performed on comparable samples from experiments with growing plants must, therefore, be limited if the samples cannot be preserved. To dry the plant material, and store it until there is ample time for analytical work is, therefore, a common procedure. Changes in the composition of the plant material may, however, take place during the process of drying. Thus, the Maillard reaction (a reaction between sugars and amines leading to loss of

Table 7. Total soluble N and amino N (estimated with ninhydrin) in distilled, cold water extracts and pH 7 buffer extracts of equivalent amounts of fresh, frozen and dried material. Total organic nitrogen of the plant material 500.3 mg/100 g dry material.

Plant material	Extracted with	Days after harvesting	mg/100 g dry matter		
			Total soluble N	Amino N 6 hrs. extraction	Amino N 22 hrs. extraction
1 g fresh stem ..	50 ml dist. water	0	124.8	37.2	35.6
1 g " " ..	50 ml pH 7 buffer	0	144.2	40.0	39.1
1 g frozen stem ..	50 ml dist. water	38	126.2	36.4	35.4
1 g " " ..	50 ml pH 7 buffer	38	144.8	41.2	40.8
0.62 g dry stem ..	50 ml dist. water	38	124.5	—	22.6
0.62 g " " ..	50 ml pH 7 buffer	38	142.8	24.2	24.7

amino N) may proceed at a fast rate under conditions similar to those of drying (Synge 1955).

In Table 6 is summarized an experiment with extraction of fresh, dried, and frozen stems and roots. Soluble dry matter, which was determined by evaporation of an aliquot of the extract, was obtained in larger quantity from dried than from fresh and frozen material. The same amount of nitrogen was extracted from all types of material. However, this does not exclude the possibility of qualitative changes in the nitrogenous compounds during drying or freezing.

Table 7 shows the results of an experiment similar to that shown in Table 6, but this time amino nitrogen too was determined in the extracts. The amount of amino nitrogen is significantly lower in the extracts of dried material than in those of fresh and frozen samples. This strongly indicates chemical changes in the amino acids during drying. The point is further elucidated by the analyses summarized in Table 8. The plant material was extracted by

Table 8. Composition of plant extracts of fresh, frozen and dried material of the same plant. Extractant pH 4 citrate buffer, no concentration of the extract prior to analysis on the column.

Material Days after harvest	Fresh 0		Frozen 32		Dried 30	
	µg N	%	µg N	%	µg N	%
Acidic and neutral amino acids ..	13.5	2.6	14.7	2.6	11.0	2.0
Amides	95.5	18.2	98.6	17.6	89.0	16.0
γ-amino butyric acid	2.9	0.6	3.3	0.6	2.0	0.4
Histidine	3.5	0.7	3.8	0.7	2.0	0.4
Lysine	0.5	0.1	0.3	0.05	—	—
"Ammonia" ca.	3.5	0.7	4.2	0.8	7.6	1.4
Arginine	368	70.1	386	68.8	291	52.3
N not accounted for		7.0		8.8		27.5
Column loads	525	100	560	100	556	100

percolation with a pH 4 citrate buffer. All the figures have been obtained by running the extract on the short column without previous concentration. The quantities of all the compounds but one were lower when the material had been dried than when kept frozen or extracted fresh. The exception is "ammonia", compound(s) at tube 125, which increased twofold as a result of drying.

It seems thus fairly well established that drying does not influence the total quantity of nitrogen extractable from the plant material. But qualitative changes in the nitrogenous compounds may occur to a great extent during drying. For analyses of the single nitrogenous compounds, extracts of fresh or frozen material should be used.

IV. Growth Experiments

The line followed in the growth experiments was to produce plants one year either extremely low or high in nitrogen, and then, during the next growing season, to follow the growth of these plant types in culture either with or without further supply of nitrogen. This, it was expected, would bring out some main effects of the previous nitrogen treatment of the plants. The method of obtaining plants with large nitrogenous reserves was founded on the hypothesis that an abundant external supply of nitrogen would have the desired effect. In one experiment the "storage volume" (*i.e.*, initial fresh weight) was also used for regulation of the amount of reserve food.

1. Plant Material

It may well be that nitrogenous reserves play a comparatively more important role in older fruit trees than in younger ones. It is also the fruiting trees which are of economic importance. However, large fruit trees are difficult to keep in culture with full control of the mineral nutrition. Further, it is difficult to obtain measurements of their production (crop + growth), and large series of analyses would have to be carried out merely to obtain a picture of the composition of one single tree. These difficulties are not met with, at least not to the same extent, in experiments with young fruit trees. Young trees seemed, therefore, to be the obvious choice for exploratory experiments on the nitrogenous reserves.

The plant material, M II rootstocks, was purchased from commercial nurseries.

In order to produce plants with different "storage volume" two sizes of rootstocks were selected the first year (1955), approximately 7 mm and 10 mm in diameter, respectively. The second year (1956) rootstocks of 8 mm in diameter were used. At planting time the plants consisted of a "stem" of quite uniform thickness. The 1955

plants had only fibrous roots spreading out from the lower part of the "stem", while the 1956 plants had root branches of several millimetres diameter from which fibrous roots spread. At the top of the "stem" there were two or more branches.

Before planting, the "stem" was marked 30 cm from the bottom end with a white paint mark. At the height of this mark the plants were budded with the variety Gravenstein in the last half of August. At the beginning of the next growing season the plants were cut down to just above the inserted bud. The main purpose of the budding was to have a well-developed bud at a certain height of the plant, from which an easily measurable maiden could develop. In this way quite uniform plants in relation to storage volume were obtained. It was thought more desirable to insert a bud at the chosen height than to depend on the development of an adventitious bud.

2. Cultivation Methods

The plants were grown in quartz sand, mostly in 12-litre enameled Mitscherlich jars; during the first year some of the plants were placed in 7-litre clay pots which had been prepared inside with a bitumen lacquer. The nutrient solution was given to the top of the jar, and superfluous moisture drained off through the hole at the bottom of the jar. At the beginning of the second year the plants were transplanted to new sand.

The plants were kept in a greenhouse without heating. Over the winter the plants were kept in the culture jars placed on the floor of the greenhouse. They were covered with a layer of sphagnum and a layer of dead leaves so as to prevent the sand from freezing.

The nutrient solution used has been derived from the Long Ashton solution (Hewitt 1952). Calcium was supplied as finely powdered carbonate. To each jar was given 10 g CaCO_3 which was mixed with the top layer of the sand at the beginning of the growing season. The other major nutrient elements were supplied in a nutrient solution of the composition given in Table 9.

Two litres of this solution were given every fortnight. The pH of the solution draining out of the jars after renewal has been in the range of 6.3 to 7, but only infrequently controlled. The amount of nitrogen supplied in the +N solution must be regarded as sufficient for optimal growth of young apple trees, according to the results of Batjer and Degman (1940).

Ferric citrate was used as a source of iron. It was added to the nutrient solution once a month in a concentration of 0.6 m.e. Fe^{+++} per litre, or it was given separately when watering was needed.

The micro-elements Mn, Zn, B, and Mo were added to the nutrient solution monthly in concentrations, and from sources as given by Hewitt (1952, Table 23).

It should be noted that plants supplied with all the necessary mineral elements grew very well in these experiments. Symptoms of mineral deficiencies or excesses were not observed. There was no sign of any other abnormality, apart from expected effects of experimental treatments.

Water was given as needed in the intervals between renewal of the nutrient solution. Tap water was used throughout the experiments, except for making up of the stock solutions of nutrient elements, where distilled water was used.

In order to counteract accumulation of salts in the culture jars, the sand was washed through with 12 litres of water for each jar once in the middle of the growing season.

Table 9. *Composition of nutrient solution of major elements.* Nitrogen only in the +N solution.

Element	m. e. per litre	Source
Mg	3	MgSO ₄ · 7H ₂ O
P	4	KH ₂ PO ₄
K	3.3	KH ₂ PO ₄ , K ₂ SO ₄ , KCl
N	10	NH ₄ NO ₃

The height of the maidens was measured at weekly or fortnightly intervals. The measurements were always taken from the lower end of the attachment between the maiden and the rootstock, to the last visible node at the tip of the maiden. The diameter of the rootstocks was measured at a fixed and marked point 20 cm from the lower end of the "stem". These measurements are referred to as "stem diameter". Measurements were made at planting, transplanting, harvesting, and sometimes during the growing season. Fresh weight determinations were also made at planting, transplanting, and harvesting.

3. Series I, 1955—56

The two-year plan for the Series I experiment is outlined in Table 10.

Total organic nitrogen in the plant material was determined at the start of the experiment. Two plants of each lot (10 and 7 mm) were taken for analysis, and divided into roots and "stem", the latter consisting of the lower 30 cm of the rootstock but with the roots cut off. The results of the analyses are given in Table 11.

According to these figures the original plant material must be characterized as poor in nitrogen when compared with the nitrogen content which has been found in comparable material at later stages of this work.

From the general inspection of the plants, their growth and appearance during the season of 1955 may be described as follows:

Table 10. *Plan of growth experiment, Series I, 1955—56.*

Stem diameter spring 1955	Nitrogen supply		Symbols	Number of plants
	First season	Second season		
10 mm	Throughout	Throughout	N/N	9
		None	N/O	9
	None	Throughout	O/N	10
		None	O/O	6
	Autumn only ¹	Throughout	n/N	8
7 mm		None	n/O	8
	Throughout	None	7 mm N/O	12

¹ From August 13th.

Table 11. *Total organic nitrogen of the plant material purchased for the experiments starting the spring of 1955. Each figure is the mean value of two determinations.*

Diameter of rootstocks mm	Total organic nitrogen mg/100 g dry material	
	Stem	Roots
10	346	1,168
7	418	1,320

The plants which received nitrogen throughout the season, grew vigorously and the leaves had a dark-green colour. Plants in culture without supply of nitrogen grew only a few shoots of a few centimetres in length, the leaves had a yellowish-green colour and fell off early in the autumn. The growth of the plants which received nitrogen from the 13th of August, was similar to that of the plants without nitrogen supply throughout the season. Thus, nitrogen supply from the middle of August did not lead to the starting of new shoot growth. The leaves of these plants began to change colour to a darker green only a few days after nitrogen application. The abscission was delayed until after frost in the autumn.

Somewhat more precise information about the effect of the different treatments in 1955 was obtained from measurements and analyses at the beginning of the 1956 season, summarized in Table 12. It is evident from the differences in stem diameter and fresh weight that the plants receiving nitrogen throughout the season made better growth than those without nitrogen supply. Nitrogen supplied from the middle of August had a very marked

Table 12. *Data of the plant material in Series I, 1955—56 at the time of transplanting (the last half of April 1956). For explanation of N treatment symbols see Table 10. The difference in stem diameter between N/O and 7 mm N/O, and 7 mm N/O and n/O is significant beyond the 1 % level. The difference in fresh weight between N/O and 7 mm N/O is significant at the 2 % level, while the difference between 7 mm N/O and n/O is significant beyond the 1 % level.*

N treatment	Number of plants	Stem diameter	Fresh weight g	mg/100 g dry plant material			
				Soluble nitrogen ¹		Non soluble nitrogen ¹	
				Stem	Roots	Stem	Roots
N/N	9	13.0	137	264	1,436	432	1,432
N/O	9	13.2	137				
O/N	10	10.9	77	27	421	242	681
O/O	6	10.4	76				
n/N	8	10.4	79	348	1,008	428	1,412
n/O	8	10.4	82				
7 mm N/O	12	11.5	112	318	912	387	1,273

¹ Mean value of two determinations.

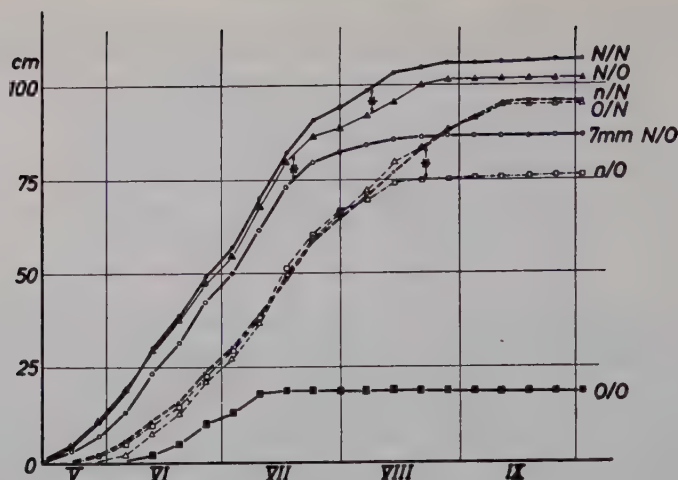


Figure 7. The 1956 growth of the maidens of plants with nitrogen nutrition controlled in 1955 and 1956. N/N: +N 1955, +N 1956; N/O: +N 1955, —N 1956; O/O: —N 1955, +N 1956; n/N: +N from the 13th of August 1955, +N 1956; n/O: +N from the 13th of August 1955, —N 1956; 7 mm N/O: +N 1955, —N 1956 (smaller plants than N/O). For further informations about the plants see Table 12. The arrows with a star show differences significant at the 5 per cent level. Months indicated by Roman numerals.

influence on the nitrogen content of the tissues, which attained the same level as in the plants supplied with nitrogen during the entire season.

The growth of the maidens through the season of 1956 is shown in Figure 7. Several points of interest are brought forward by this figure. There is a clear effect on the plant growth of the previous year's nitrogen treatment. Plants supplied with nitrogen in 1955, but grown without nitrogen in 1956 (N/O and n/O) kept the same height, for quite some time of the growing season, as plants which received nitrogen both years (N/N and n/N). Plants without supply of nitrogen during both years (O/O) produced only small maidens at the beginning of the season. The current year's nitrogen supply had only a marked effect on the length growth after the best months for vegetative growth had passed (June, July and part of, or the whole of August) if the plants had received nitrogen the previous year. Plants which received nitrogen throughout the previous season (N/N, N/O, 7 mm N/O), started new shoot growth significantly earlier in the spring than those without nitrogen supply (O/N, O/O) or those with supply only in the autumn (n/N, n/O), irrespective of the current year's nitrogen treatment. A point of interest may also be that for plants supplied with nitrogen during the current year, growth terminated earlier in the autumn in those plants which had received nitrogen throughout the previous season (N/N) than in those

without nitrogen (O/N) or in those supplied with nitrogen only in the autumn (n/N). The final height of plants receiving nitrogen in 1955, but grown in nitrogen-free culture in 1956, may have been limited by the amount of stored nitrogen. This is indicated by the correspondence between final height of maidens and the initial fresh weight ("storage volume") to be found in Table 12.

4. Height-Weight Relationship

The height of the maidens is taken as an index of the production of the plants. Arguments may be raised against this, because plant production should rather be measured by fresh weight or dry weight increase. In this experiment the plants were harvested at intervals through the growing season. Then both fresh and dry weight of the maidens were taken. Height measurements were taken at weekly intervals. These data furnished material for a statistical study of the relationship between height and fresh and dry weight respectively. The results of these studies are given in Figures 8 and 9.

In Figure 8 the height of the maidens is plotted against fresh weight, and against the common logarithm of the fresh weight. The relationship between height and fresh weight is of an exponential type. Regression and correlation data for the relationship between height and logarithm of fresh weight are compiled in Table 13. The data was analysed according to Snedecor (1948).

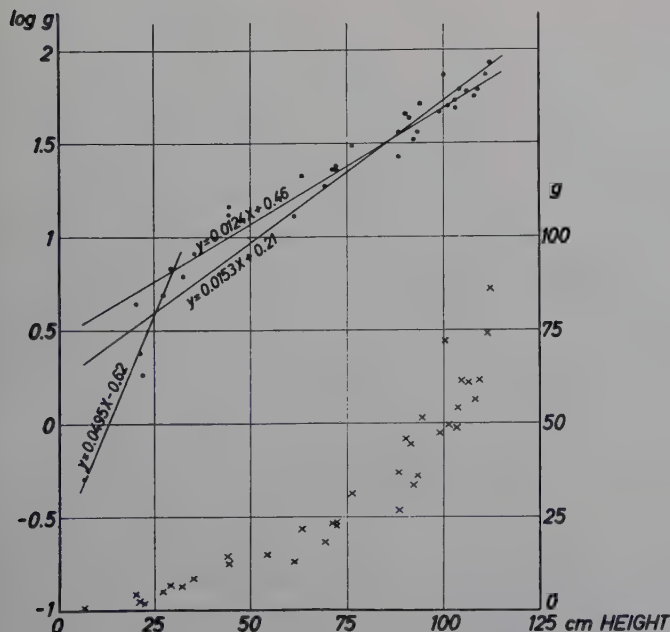


Figure 8. Relationship between the height and the fresh weight of the maidens. \times = fresh weight g; \bullet = log fresh weight g.

Table 13. Regression and correlation data for height of maiden and log. fresh weight.

N treatment	Correlation coefficients	Regression coefficients	Constants	Errors of estimate	
				Sum of squares	d. f.
N/N	0.987	0.01099	0.61	0.00723	5
N/O	0.984	0.01046	0.65	0.00636	4
O/N	0.994	0.01332	0.41	0.00930	4
O/O	0.909	0.04956	— 0.62	0.12286	3
n/N	0.933	0.01275	0.47	0.07602	3
n/O	0.974	0.01338	0.41	0.03542	5
Total	0.951	0.01532	0.21		34
Total without O/O	0.974	0.01247	0.46		29

As is apparent, there are significant differences at the 1 % level among the group regression coefficients, if all the six treatments are analysed together. From the separate regression coefficients, it is seen that the coefficient of the O/O group is strikingly different from all the others. If the O/O group is withdrawn from the material, a new analysis shows that significant differences do not occur between the remaining coefficients.

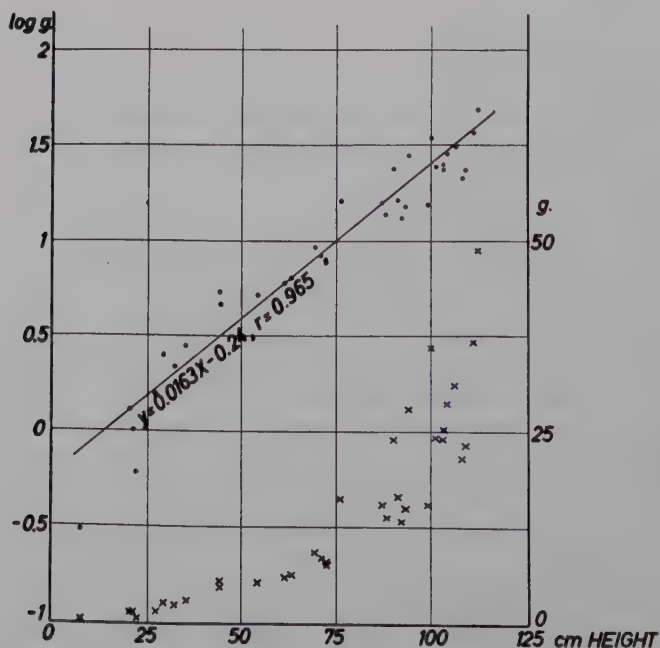


Figure 9. Relationship between the height and the dry weight of the maidens. \times = dry weight g; \bullet = log dry weight g.

This analysis would indicate that the height of the maidens may be used as a fairly precise substitute for the fresh weight. It is also clear that no great error is introduced if the same conversion factors are used for all the plants, except for the nitrogen-starved ones, in order to obtain a picture of the fresh weight from the height measurements.

Figure 9 shows a close logarithmic relationship between height of the maidens and the dry matter. The result supports the idea that the increase in height can be used as a fairly reliable measure of the production of these plants.

A further argument which may be raised against the use of the height of the maiden as an index of production, is that measurements are made only on part of the growing plant. Nitrogenous fertilizers, in general, increase particularly growth of the top as compared with the roots (Miller 1938). For apple trees in particular it has been shown by Blake *et al.* (1937) that a low level of nitrogen is associated with the development of a relatively extensive root-system. If this holds true also when the plants are able to grow, apparently normally, on nitrogenous reserve food, the omission of measurements of root growth in these experiments would lead to an underestimation of the importance of the reserves for plant production as a whole.

5. Series II, 1956—57

As a supplement to the 1955/56 growth experiment, the conditions and results of a similar experiment lasting over the seasons 1956 and 1957 are shown below. Nitrogen treatment was as given in Table 14.

The plants supplied with nitrogen in 1956 did not grow as well as the plants with the same treatment in 1955, whereas those without supply of nitrogen made a fairly good growth and had darker green leaves than comparable plants of the Series I.

The plant material was analysed for soluble and non-soluble nitrogen at the beginning of each season. The results are given in Table 15.

The plants of this series had a higher nitrogen content from the start than

Table 14. *Plan of growth experiment, Series II, 1956—57.*

Stem diameter spring 1956	Nitrogen supply		Symbols	Number of plants
	First season	Second season		
8 mm	Throughout	Throughout	N/N	6
		None	N/O	6
	None	Throughout	O/N	6
		None	O/O	6

Table 15. *Data of the plant material in the Series II, 1956—57 at the time of planting (56) and transplanting (57).*

Plant material reference	Sampling date	Stem diameter mm	Fresh weight g	mg/100 g dry plant material			
				Soluble nitrogen ¹		Non soluble nitrogen ¹	
				Stem	Roots	Stem	Roots
Purchased rootstocks	29/5 -56	8.1		418	658	421	973
N/N and N/O	16/5 -57	10.2	128	568	1,174	475	1,008
O/N and O/O	16/5 -57	9.8	104	211	422	300	1,008

¹ Mean value of two determinations.

those of Series I. This may be the reason why these plants, grown for one season without nitrogen, were more abundant in nitrogen than the comparable plants of Series I. The high content of soluble nitrogen in the O/— plants at the beginning of the second season is noticeable. This would indicate that plants fairly rich in nitrogen may not be exhausted in one growing season even when grown without external supply of nitrogen. The autumnal migration of nitrogenous compounds from the leaves to the younger parts of the branches may be a process of importance in this connection.

The growth of the maidens in the second experiment is demonstrated by Figure 10. The maidens developed in general agreement with those of the 1955/56 experiment, only that smaller differences were found between the plants of the different nitrogen treatments. This may be due to the fact that the attempt to produce plants with large differences in nitrogen status was less successful in this experiment than in Series I.

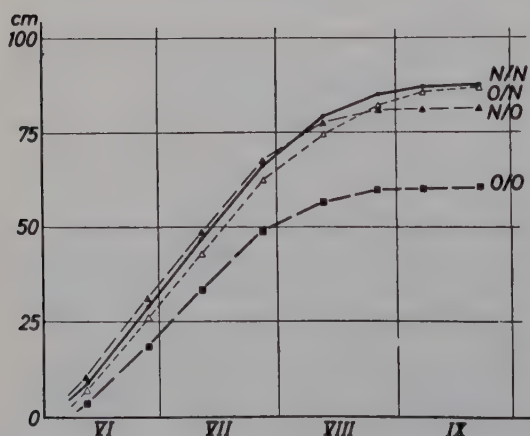


Figure 10. *The 1957 growth of the maidens of plants with nitrogen nutrition controlled in 1956 and 1957. N/N: +N 1956, +N 1957; N/O: +N 1956, —N 1957; O/N: —N 1956, +N 1957; O/O: —N 1956, —N 1957. Months indicated by Roman numerals.*

V. Changes in Soluble and Non-soluble Nitrogen During the Growth Season

The object of the chemical part of the program was mainly to investigate changes in the nitrogenous composition of the plants as affected by nitrogen treatment and growth. Material for nitrogen analyses was taken at different times during the growth season in the last year of the Series I growth experiment. The plants were divided into four parts, roots, "stem", maiden and leaves, and each part analysed separately.

The roots and the "stem" are to be considered, *a priori*, as the storage organs in this experiment. New tissues are, however, added to these organs also during the growth season.

1. "Stems"

Figure 11 shows the changes of soluble and non-soluble nitrogen in the "stem" of the plants which received nitrogen throughout the season of 1955.

The general picture given by Figure 11, is that the non-soluble nitrogen was maintained at a fairly constant level throughout the season, whereas the soluble fraction declined rapidly from a high level at the beginning of the season, to a very low one from August and onwards. This points to the function of the initial pool of soluble nitrogenous compounds as reserve food.

The curves further show a significant increase in the soluble fraction from the middle of April to the last half of May, concurrent with a decrease of the non-soluble fraction. This occurs irrespective of the current year's nitrogen treatment. The latter data of analysis correspond to the starting time of the current year's growth. This phenomenon should probably be interpreted as protein breakdown occurring at the time of, or just prior to the start of new growth. A similar aspect of the nitrogen metabolism has been found by Rahn (1932) in other plant species, and interpreted as a mobilization of reserve proteins.

The "stems" of plants receiving nitrogen in the second season were somewhat higher in both soluble and non-soluble nitrogen throughout the season, than the "stems" of plants which were not supplied with nitrogen in 1956. However, in both plant groups practically the entire soluble nitrogen fraction of the "stem" was apparently re-distributed either to new tissue of the "stem" or to other plant parts. This indicates that the building up or even the maintenance of nitrogenous reserves is not a straightforward question of nitrogen supply.

Figure 12 corresponds to Figure 11, except that it represents plants that were grown without supply of nitrogen the previous season (O/N and O/O).

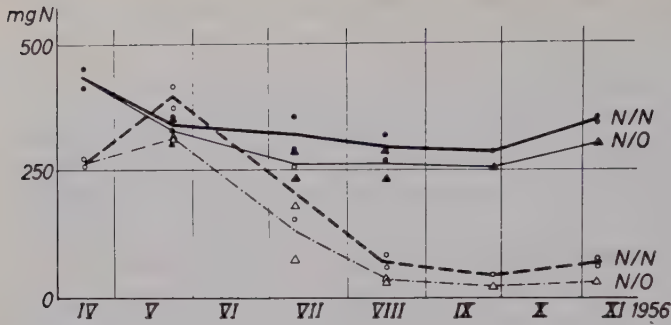


Figure 11. Soluble N (broken lines) and non-soluble N (unbroken lines) in the stem of plants receiving nitrogen throughout the 1955 season. N/N: +N 1955, +N 1956; N/O: +N 1955, —N 1956. On the ordinate mg N per 100 g of dry plant material. Months indicated by Roman numerals.

An increase in soluble nitrogen at the expense of non-soluble nitrogen prior to new growth is demonstrated at a significant level by the O/O curves. The much larger increase in soluble nitrogen which occurred in the O/N plants during the first month after transplanting cannot be fully explained as a result of protein breakdown. Obviously, these plants must have absorbed nitrogen from the medium before growth started. Since this absorption can be demonstrated by analysis of the "stem" only, it shows not only that nitrogen has been absorbed, but also that it has been translocated upwards.

The plants supplied with nitrogen not only showed an increase in soluble nitrogen prior to new growth, but kept a fairly high level of this fraction throughout the season. At the same time the non-soluble fraction showed an increasing trend. The rate of growth of these O/N plants was comparable to that of the N/N plants. Altogether this would indicate a fairly vigorous absorption of nitrogen by the O/N plants in contrast to that of the N/N group. Thus, the previous year's nitrogen supply may be a factor influencing the current year's nitrogen absorption.

Figure 13 shows the analytical results of "stems" which received nitrogen from the middle of August in the previous year (n/N and n/O). The general

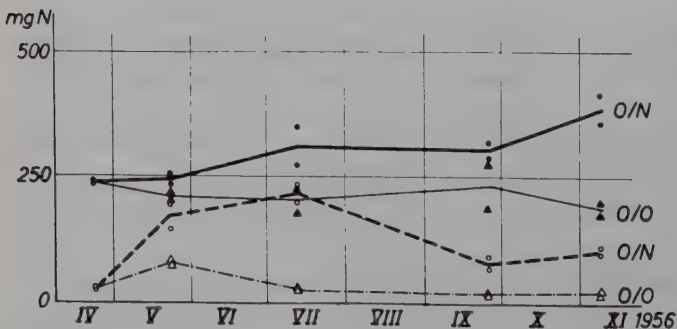
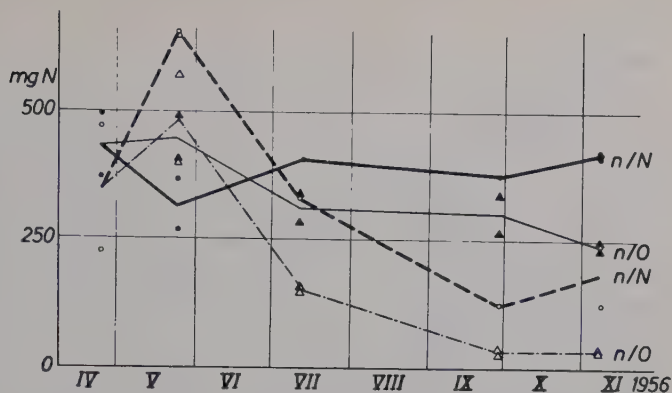


Figure 12. Soluble N (broken lines) and non-soluble N (unbroken lines) in the stem of plants grown through the season of 1955 without supply of nitrogen. O/N: —N 1955, +N 1956; O/O: —N 1955, —N 1956. Coordinates as in Figure 11.

Figure 13. Soluble N (broken lines) and non-soluble N (unbroken lines) in the stem of plants grown with supply of nitrogen from the 13th of August 1955. n/N: +N autumn 1955. +N 1956; n/O: +N autumn 1955, —N 1956. Coordinates as in Figure 11.



trend of both the soluble and the non-soluble fraction of the n/O plants follows the corresponding curves for the N/O plants (presented in Figure 11). The plants receiving nitrogen during the current year (n/N) are marked by a much higher level of both soluble and non-soluble nitrogen than those of the non-nitrogen treatment (n/O). This indicates nitrogen absorption, and in this respect these plants (n/N) behaved similarly to those without nitrogen throughout the previous season (O/N).

The "stem" data seem to give a probable explanation of a somewhat surprising fact mentioned above, *viz.*, that of all the plants supplied with nitrogen during 1956, those which received nitrogen through the entire previous season (N/N) terminated growth earlier in the autumn than the plants of other treatments (O/N and n/N). As seen from Figures 11—13, the 1956 autumn-level of soluble nitrogen was markedly lower in the N/N plants than in those supplied with less or no nitrogen during the previous season. Apparently, growth must be related to the soluble nitrogen pool, which cannot always be increased by external supply.

2. Roots

The results of root-tissue analyses are presented in Figures 14, 15 and 16. The figures are arranged in the same order as the "stem" series with respect to the previous year's nitrogen treatment.

The soluble fraction of the roots varied through the season in a similar manner to the fraction in the corresponding "stems". Special mention should be made of the depletion of soluble nitrogen in the roots of the plants receiving nitrogen throughout both growing seasons (N/N), as compared to the plants of the two other nitrogen pre-treatments (O/N and n/N).

The non-soluble fraction of the roots shows an aspect of the nitrogen metabolism that is not apparent from the analytical results of the "stems".

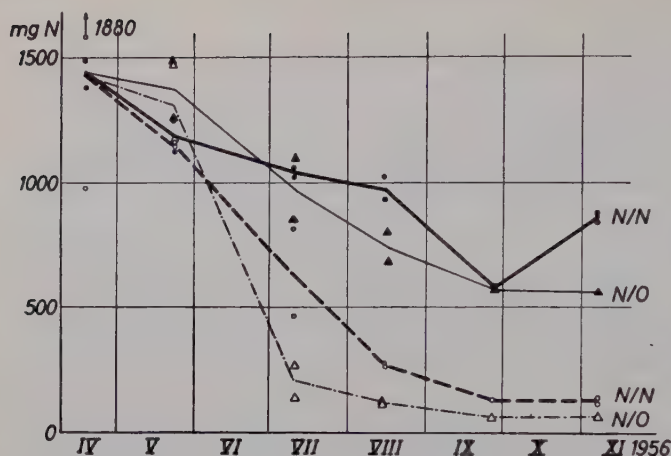


Figure 14. Soluble N (broken lines) and non-soluble N (unbroken lines) in the roots of plants receiving nitrogen throughout the 1955 season. N/N: +N 1955, +N 1956; N/O: +N 1955, —N 1956. Coordinates as in Figure 11.

There is a decrease in the non-soluble nitrogen throughout the growing season in the root tissue of the plants which received nitrogen the previous season. This fraction in the "stem" only showed a decrease at the beginning of the season, and after nitrogen application an increase apparently took place later in the season. The non-soluble nitrogen in the roots of plants without nitrogen supply during both seasons was kept at a fairly constant level throughout the season. These results seem to indicate that non-soluble nitrogen may be quantitatively more significant as a nitrogenous reserve than was indicated by the mobilization of non-soluble nitrogen in the "stem" at the beginning of the season.

When roots are collected from time to time throughout a growing season

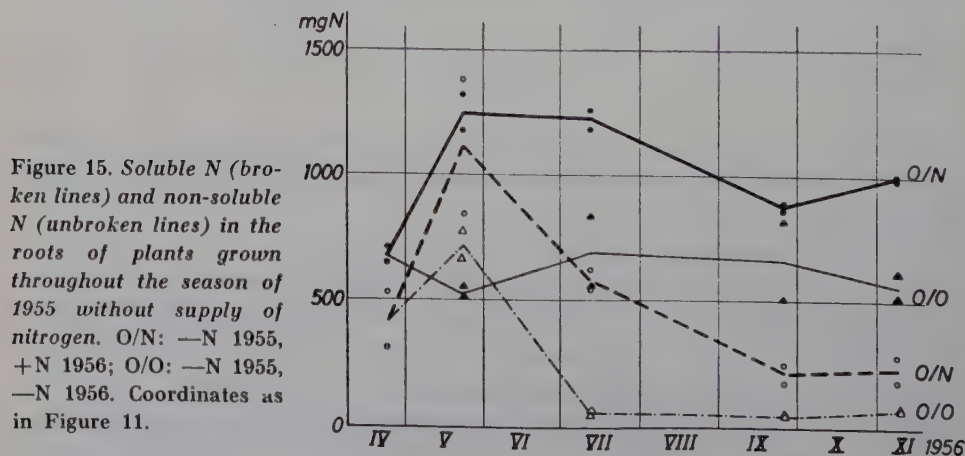


Figure 15. Soluble N (broken lines) and non-soluble N (unbroken lines) in the roots of plants grown throughout the season of 1955 without supply of nitrogen. O/N: —N 1955, +N 1956; O/O: —N 1955, —N 1956. Coordinates as in Figure 11.

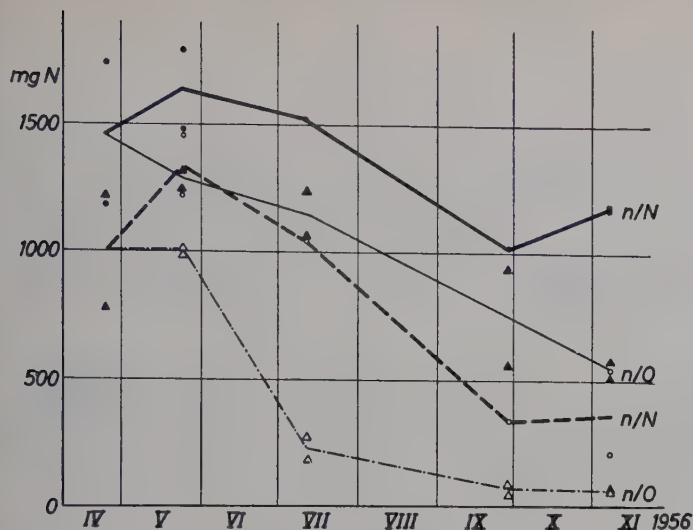


Figure 16. Soluble N (broken lines) and non-soluble N (unbroken lines) in the roots of plants grown with supply of nitrogen from the 15th of August 1955. n/N: +N autumn 1955, +N 1956; n/O: +N autumn 1955, —N 1956. Coordinates as in Figure 11.

the subsequent samples show increasing amounts of new tissue. New tissues are generally considered richer in nitrogen, particularly in protein nitrogen, than older tissues. Therefore, growth during the sampling period would be expected to give a rise in the percentage of non-soluble nitrogen in subsequent samples. A downward trend in the non-soluble nitrogen must thus be interpreted as a re-distribution of this fraction. The role of the non-soluble fraction as nitrogenous reserve food might be underestimated if the possible effect of new growth is not taken into consideration.

3. Maidens

The maidens are current year's growth, and are thus not to be looked upon as storage organs when the apple trees are considered as perennial plants.

Figure 17 gives the results of the analyses of maidens of plants which received nitrogen throughout the previous season. The maidens were fairly rich in soluble nitrogen at the beginning of the growing season, which indicates a vigorous translocation of stored nitrogen at the time when the storage organs have plenty of available material. Later on the amount of soluble nitrogen diminished in correspondence with the seasonal falling trend of soluble nitrogen in the storage organs. The nitrogen level of the maidens which received nitrogen throughout the previous season was markedly increased by the current external supply, especially at the beginning of the growing season, while little or no increase was shown by the "stem" and roots. This indicates that nitrogen may be absorbed and utilized for active growth under conditions unfavourable for accumulation in storage organs.

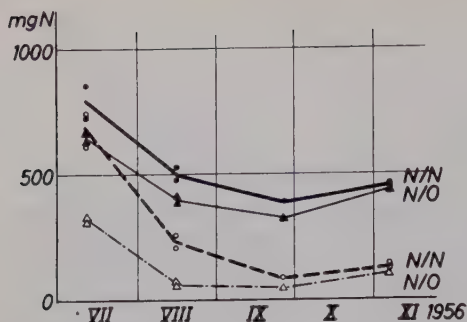


Figure 17. Soluble N (broken lines) and non-soluble N (unbroken lines) in the maidens of plants receiving nitrogen throughout the 1955 season. N/N: +N 1955, +N 1956; N/O: +N 1955, —N 1956. Coordinates as in Figure 11.

Growth of the maidens went on at the same high rate during early summer, irrespective of the differences in external supply and in internal nitrogen level, until the soluble fraction had declined to a comparatively low level. Thus, the internal concentration of soluble nitrogen required for good growth is apparently not very high. It might be argued that a high nitrogen content could be maintained in the apical part of the maidens without being detected by these analyses. However, the apices of other plants have been shown to be relatively low in soluble nitrogen substances and free amino acids (Allsopp 1948). Besides, it seems very unlikely that the lower parts of the maidens could have been more depleted than the "stems".

Figure 18 represents the plants which did receive nitrogen the previous season (O/O and O/N). Without further supply of nitrogen the maidens kept a fairly constant level of both soluble and non-soluble nitrogen throughout the season. The current year's nitrogen supply had a marked effect on the level of both soluble and non-soluble nitrogen, particularly at the beginning of the season.

Figure 19, which shows the results of the analyses of maidens from plants receiving nitrogen only from the middle of August the previous year, chiefly presents the same pattern as Figure 17, which represents plants with nitrogen supply throughout the previous season.

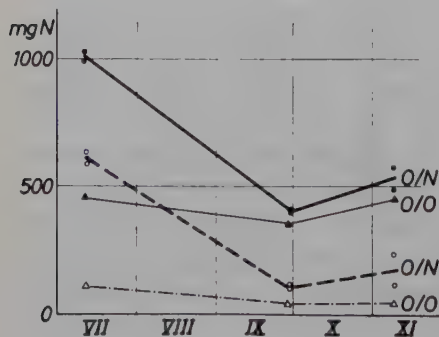
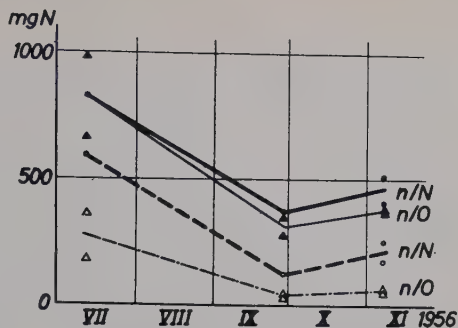


Figure 18. Soluble N (broken lines) and non-soluble N (unbroken lines) in the maidens of plants grown without supply of nitrogen through the 1955 season. O/N: —N 1955, +N 1956; O/O: —N 1955, —N 1956. Coordinates as in Figure 11.

Figure 19. Soluble N (broken lines) and non-soluble N (unbroken lines) in the maidens of plants supplied with nitrogen from the 13th of August 1955. n/N: +N autumn 1955, +N 1956; n/O: +N autumn 1955, —N 1956. Coordinates as in Figure 11.



Maidens which stopped growth early in the autumn, even when supplied with nitrogen, show a lower content of soluble nitrogen than those which did not stop growing until later (cf. Figures 7, 18 and 19).

4. Leaves

The results of the chemical analyses of the leaves of the Series I plants are presented in Figures 20, 21 and 22.

These figures show features distinct from those representing the other plant parts.

The first clear and contrasting observation is that the soluble nitrogen is showing a fairly constant level throughout the season. Thus, the high spring level of soluble nitrogen of the other plant parts was not conveyed to the leaves.

In the roots, "stems" and maidens the effect of the current year's nitrogen treatment was often most apparent in the soluble fraction. This was not the

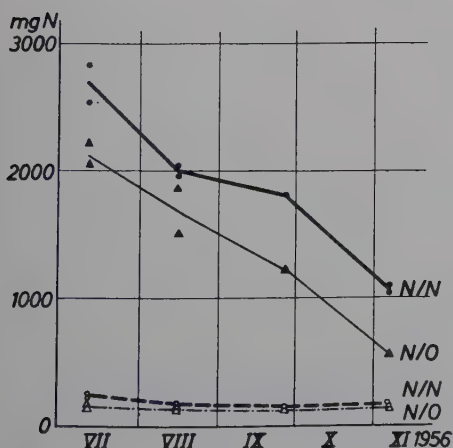


Figure 20. Soluble N (broken lines) and non-soluble N (unbroken lines) in the leaves of plants receiving nitrogen throughout the season of 1955. N/N: +N 1955, +N 1956; N/O: +N 1955, —N 1956. Coordinates as in Figure 11.

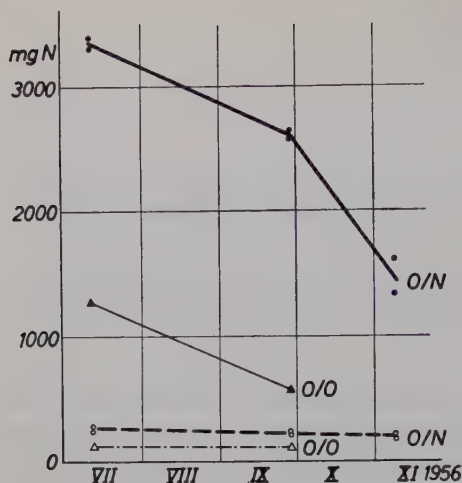


Figure 21. Soluble N (broken lines) and non-soluble N (unbroken lines) in the leaves of plants grown without supply of nitrogen through the season of 1955. O/N: —N 1955, +N 1956; O/O: —N 1955, —N 1956. Coordinates as in Figure 11.

case with the leaves. Here the current nitrogen supply had a very marked effect on the non-soluble fraction. Further, the current year's supply of nitrogen led to a significantly higher level of nitrogen in the leaves of plants without previous supply, or with supply of nitrogen from the middle of August, as compared to plants which received nitrogen throughout the previous season.

It is apparent that the nitrogen level is not always immediately related to the amount of externally available nitrogen. Thus the previous year's nitrogen treatment appears to influence the current absorption of nitrogen. It may, however, be a factor associated with the previous year's nitrogen treatment which is directly responsible for this effect.

It has been shown by Ljones (1951 and 1954) and Oland (1955) that the

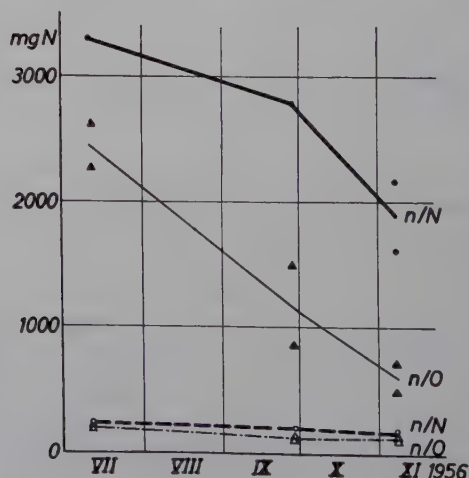


Figure 22. Soluble N (broken lines) and non-soluble N (unbroken lines) in the leaves of plants grown with supply of nitrogen from the 13th of August 1955. n/N: +N autumn 1955, +N 1956; n/O: +N autumn 1955, —N 1956. Coordinates as in Figure 11.

content of the total organic nitrogen in shoot leaves of trees with biennial bearing is higher in the cropping year than in the non-cropping year. The data presented above show that the nitrogen content of the leaves, as well as that of the entire plant, was lower in plants which had received nitrogen and had had a vigorous growth the previous year, than in plants which only showed a poor growth, resulting either from no supply of nitrogen or from supply of nitrogen in the autumn exclusively. These two observations may be explained by assuming that vigorous growth as well as heavy cropping may exhaust the plant of carbohydrate. This assumption is in agreement with the sequence of dominance, fruits—apical buds—lateral buds—roots, which according to Arisz (1952) is obtained in the competition for foodstuffs. Absorption of nitrogen and synthesis of organic nitrogen compounds does supposedly take place at the expense of carbohydrates already stored in the roots, and is only to a limited extent dependant on simultaneous supply. The required carbohydrate reserves would be absent after a year of heavy cropping as well as after vigorous growth.

VI. Reserve Nitrogen

1. *Relative Role of Soluble and Non-soluble Fraction*

The seasonal trends of the two nitrogen fractions in the roots have already indicated that part of the non-soluble nitrogen may serve as nitrogenous reserve food. The increase of soluble nitrogen occurring at the expense of the non-soluble fraction at the time of the budbreak was particularly evident. However, all these data were obtained from gross analyses of the entire organs. Obviously, the effects of internal nitrogen metabolism, growth, and re-distribution between the different tissues could not be disentangled from such data. In order to get a clearer picture of the nitrogen balance, it was thought desirable to follow the changes occurring during the growth period in a typical non-growing storage tissue, *viz.*, old wood of the "stem".

Some of the Series I plants were set apart for this experiment. The plants were divided into two groups which were given reversed nitrogen treatments during the two consecutive seasons. Material for analyses was taken during the second season, at the start of the growth period and as soon as growth had ceased in the autumn.

The old wood was separated from the bark in the spring samples, and from bark and new wood in the autumn samples.

Table 16 gives information on the analysed plant material. A slight indication is found that the roots of plants with no nitrogen supply during the current year have increased more in dry weight than have the roots of those

Table 16. *Stem diameter and dry weight of the different plant parts used for analysis.*

N treatment	Plant No.	Sampling date	Stem diameter		g dry weight					
			Spring mm	Autumn mm	Roots	Stem			Maiden	Leaves
						Old wood	New wood	Bark		
O/—	94	16/5 -56	12.1		4.4	17.5		4.4		
"	100	16/5 -56	11.5		5.1	15.8		4.7		
O/N	36	28/9 -56	11.4	16.0	21.5	14.9	18.8	7.0	37.7	20.2
"	41	28/9 -56	12.9	17.2	18.1	16.4	20.3	9.6	28.5	17.9
N/—	114	8/5 -56	11.2		17.9	15.5		4.2		
"	119	8/5 -56	11.5		18.3	15.1		4.0		
N/O	63	29/8 -56	12.4	15.4	36.9	18.7	11.2	9.1	15.0	15.7
"	64	29/8 -56	11.6	14.1	32.2	15.0	11.6	7.5	15.8	16.2

with nitrogen supply. Here the difference in time of sampling should be borne in mind, and also the difference in original root-weight.

Table 17 gives the amount of non-soluble nitrogen in the analysed plant material. In the old wood of the N/O plants a very pronounced decrease in this fraction occurred during the growth season. The figures for the parallel O/N group seem to indicate a slight increase, although the difference is not statistically significant. These figures suggest that quite a large part of the non-soluble nitrogen may serve as nitrogenous reserve food.

The amount of soluble nitrogen in the plant parts of this experiment is given in Table 18. If the difference between spring and autumn figures in this fraction are compared with the corresponding difference in the non-soluble fraction (Table 17), it becomes apparent that the soluble nitrogen may be quantitatively the most important nitrogenous reserve in the stem wood.

A conspicuous point in this latter table is the large amount of soluble nitrogen present in the spring in the bark of plants grown without nitrogen supply the previous season. This may be taken to indicate mobilization of

Table 17. *Non-soluble nitrogen.*

N treatment	Plant No.	Sampling date	mg N/100 g dry plant material					
			Roots	Old wood	New wood	Stem bark	Maiden	Leaves
O/—	94	16/5 -56	714	147		637		
	100	16/5 -56	683	110		510		
O/N	36	28/9 -56	858	159	261	690	401	2.649
	41	28/9 -56	895	154	256	718	401	2.576
N/—	114	8/5 -56	1,350	221		991		
	119	8/5 -56	1,195	199		958		
N/O	63	29/8 -56	575	122	194	524	284	1.090
	64	29/8 -56	543	121	160	521	285	1.089

Table 18. *Total soluble nitrogen.*

N treatment	Plant No.	Sampling date	mg N/100 g dry plant material					
			Roots	Old wood	New wood	Stem bark	Maiden	Leaves
O/—	94	16/5 -56	777	50		212		
	100	16/5 -56	667	49		145		
O/N	36	28/9 -56	176	42	77	76	111	203
	41	28/9 -56	247	73	119	88	103	228
N/—	114	8/5 -56	998	286		644		
	119	8/5 -56	827	216		529		
N/O	63	29/8 -56	27	19	31	65	41	114
	64	29/8 -56	79	22	24	74	54	125

reserves in a comparatively active metabolizing tissue at the start of a season's growth. The comparatively low quantity of soluble nitrogen in the bark tissue in the autumn, in spite of nitrogen supplied to the plants, seems to underline the reserve role of the soluble nitrogen found in this tissue in the spring. In peach trees Nasharty (1944) found a notable increase in the nitrogen content of shoot bark before blossoming, which was taken as an indication of extensive upward movement of nitrogen before transpiration sets in.

Although the above experiment can only be looked upon as a small fraction of the Series I experiment, the data obtained verify several points of the results discussed earlier.

Additional information on the reserve function of the non-soluble nitrogen may be obtained from the analyses of a number of plants from Series II, where the experimental treatments were extended beyond those of the experiment reported in Tables 16, 17, and 18. In the present context only the data concerning non-soluble nitrogen in old wood need be mentioned. For convenience the mean values for the different pairs of plants are given here in Table 19 (*cf.* Table 20).

The new feature which becomes apparent from these data is the even level of the non-soluble nitrogen in the old wood following many of the treatments. Plants grown without nitrogen the previous season show the same level of non-soluble nitrogen at the end of the current year as at the beginning, irrespective of the current year's nitrogen treatment. When grown without supply of nitrogen, the plants with a high content in the spring show a lowering of the amount of non-soluble nitrogen to a level equal to that of plants grown without supply of nitrogen for two seasons. A marked decrease in the content of non-soluble nitrogen was found in these plants even when grown with supply of nitrogen.

These facts underline the true reserve role of a part of the non-soluble fraction. It may well be that the non-soluble reserve nitrogen is a fraction

Table 19. *Non-soluble nitrogen in old wood. Mg N/100 g dry plant material.*

N-supply 1956	Spring 1957	N-supply 1957	Autumn 1957
+ N	223	+ N	182
— N	147	— N	144
		+ N	159
		— N	146

with its own characteristics distinct from other non-soluble nitrogen. Further, the quantity of non-soluble nitrogen needed for the normal functions of the plant may be quite a stable fraction, at least in some older tissues.

2. Composition of the Soluble Fraction

The standard method for obtaining the soluble nitrogen has in this work been to extract a small portion of plant material by a 0.2 *M* NaCl extractant, buffered with phosphate to pH 7. Several other extractants and extraction procedures yielded the same amount of soluble nitrogen. Most of the nitrogen obtained in the extracts dialysed against water through a cellophane membrane, and it was concluded that the soluble fraction would mainly comprise low-molecular weight nitrogen compounds. It seemed, therefore, likely that the Moore and Stein (1951) method afforded a means of obtaining information on the quantitative composition of the soluble fraction.

Plant material was obtained from the Series II, 1956—57. The material was divided into roots, bark (of "stem"), old wood, and new wood, if formed, and kept, until analysed, in a freeze-box at -25°C .

The growth of the maidens has been shown in Figure 10. The results of the analyses are given in Table 20.

First of all attention should be drawn to the varying amount of arginine in plants of different nutritional status. In the spring the nitrogen of arginine amounts to 70 per cent or more of the soluble fraction in the wood of plants which received nitrogen during the previous season. The plants (Nos. 56, and 70) must be characterized as rich in nitrogen. After plants of this same group had grown a maiden of more than 80 cm in height without supply of nitrogen, all the analysed plant parts were low in arginine, and only traces of arginine remained in the old wood (Nos. 15, and 16). All other analysed samples with a comparatively high total nitrogen content yielded a soluble fraction with a high percentage of arginine, whereas samples low in nitrogen contained little arginine. The decrease in nitrogen content of the different

Table 20. Soluble and non-soluble nitrogen of different parts of plants grown under different nitrogen treatments, and data on the composition of the soluble fraction.

N treatment	Plant No.	Sampling date	Plant part	mg N/100 g dry weight		Total N in the compounds as % of soluble N					Per cent of soluble N accounted for
				Non-soluble	Soluble	Acidic + neutral amino acids	Amides	γ-amino butyric acid	Histidine	Arginine	
N/—	56	16/5 -57	wood	214	379	2.1	17.0	1.5	1.8	76.0	98.4
"	"	"	bark	928	678	6.7	26.4	1.4	1.1	60.0	95.6
"	"	"	roots	917	800	3.8	45.0	1.4	1.1	46.2	97.5
"	70	"	wood	233	502	2.5	17.0	1.1	1.7	70.0	92.3
"	"	"	bark	1,062	903	4.6	25.0	1.2	0.7	60.6	92.1
"	"	"	roots	1,096	1,547	4.7	43.0	1.0	1.2	49.6	99.5
O/—	9	"	wood	156	177	4.7	33.1	1.3	2.0	50.3	91.4
"	"	"	bark	690	270	9.5	39.1	1.8	2.4	38.4	91.2
"	"	"	roots	1,003	494	5.4	48.3	1.1	0.6	31.9	87.4
"	26	"	wood	139	170	3.3	34.7	1.6	2.4	54.5	96.5
"	"	"	bark	677	283	7.2	40.8	1.7	2.6	42.8	95.1
"	"	"	roots	1,013	350	6.4	45.2	1.1	0.4	23.0	76.1 ¹
N/N	22	25/9 -57	old wood	174	177	2.4	41.2	1.6	0.6	49.4	95.2
"	"	"	new wood	320	301	2.2	37.3	1.5	0.7	55.0	96.7
"	"	"	bark	803	369	3.1	55.7	1.1	0.6	28.9	89.4
"	"	"	roots	899	731	2.3	60.9	0.9	0.6	25.5	90.2
"	23	"	old wood	190	264	2.2	32.1	0.6	0.7	55.2	90.8
"	"	"	new wood	287	375	2.1	27.0	0.9	0.7	62.0	92.7
"	"	"	bark	781	351	2.6	47.8	1.1	0.4	35.6	87.5
"	"	"	roots	899	777	3.1	51.9	0.6	0.8	37.2	93.6
N/O	15	"	old wood	141	18	12.6	27.0	3.4	0.8	traces	43.8
"	"	"	new wood	297	37	10.7	25.2	3.9	1.6	2.2	43.6
"	"	"	bark	643	74	12.4	22.7	3.7	1.1	5.8	45.7
"	"	"	roots	642	81	11.2	19.7	2.9	1.4	8.5	43.7
"	16	"	old wood	148	20	13.9	26.7	3.8	2.4	2.6	49.4
"	"	"	new wood	241	37	10.7	22.4	4.0	2.5	8.7	48.3
"	"	"	bark	619	48	13.3	23.9	3.4	1.6	3.0	45.2
"	"	"	roots	666	85	13.7	20.3	3.2	2.4	6.9	46.5
O/N	7	16/8 -57	old wood	147	91	5.7	53.2	1.7	0.8	30.4	91.8
"	"	"	new wood	305	201	5.9	38.3	2.0	1.4	48.2	95.8
"	"	"	bark	695	167	5.8	54.9	2.8	1.1	26.7	91.3
"	"	"	roots	1,165	434	5.9	60.8	1.9	1.0	21.4	90.9
"	8	"	old wood	171	141	5.1	39.4	1.8	0.6	50.0	96.9
"	"	"	new wood	324	251	6.4	34.7	1.9	1.3	50.3	94.6
"	"	"	bark	691	210	6.8	51.3	2.0	1.4	27.2	88.7
"	"	"	roots	1,131	505	5.7	55.3	1.9	1.2	24.5	88.6
O/O	3	"	old wood	147	18	14.6	27.0	2.6	1.0	5.2	50.4
"	"	"	bark	468	37	10.7	18.4	2.0	1.0	2.7	34.8
"	"	"	roots	676	96	11.4	26.5	2.9	1.3	4.0	46.1
"	4	"	old wood	146	17	12.8	23.0	3.4	1.0	5.9	46.2
"	"	"	bark	443	49	10.0	20.3	2.3	0.9	3.1	36.6
"	"	"	roots	692	91	13.6	24.2	2.7	1.3	2.8	44.6

¹ Prominent ammonia peak.

tissues was accompanied by new growth. Thus, arginine is revealed as an important constituent of the nitrogenous reserves.

The arginine content was higher in the wood than in the bark. This is consistent with the findings of Mulay (1932) that basic nitrogen (most likely for the main part arginine) constitutes a much higher fraction in the wood than in the bark of the non-protein nitrogen of shoots of the Bartlett pear.

The percentage of nitrogen in amides was comparatively high in the extracts of all plants and plant parts. An inverse relationship between per cent nitrogen in arginine and amides was often apparent, particularly in the nitrogen-rich samples. The quantity of amides distributed from the storage tissues during new growth was, however, considerable, because of the large decrease in the total soluble nitrogen of which the amides constituted a comparatively constant part. In the extracts of some tissues, particularly from the roots, the percentage of nitrogen in amides had also decreased considerably during growth.

The percentage of nitrogen accounted for in the acidic and neutral amino acids was lower in extracts of plants rich in soluble nitrogen than in plants with a low content of soluble nitrogen. The same was the case with γ -amino butyric acid. This must be taken to indicate that these compounds do not act as specific reserve compounds.

More than 90 per cent of the nitrogen was accounted for in the extracts of plants with a high content of soluble nitrogen, whereas only about 40 to 50 per cent of the soluble nitrogen of nitrogen-poor plants was accounted for. A constant error might be responsible for this difference, but indications pointing in this direction were not found during the analytical work. It seems more likely that some unidentified nitrogen compound was extracted together with the free amino acids. Although this substance might only account for a small percentage of the total nitrogen in plants with ample nitrogenous reserves, it might constitute a much greater part in the extracts of plants depleted of the reserve. This hypothesis implies that the plants do not utilize the unknown substance during growth as a nitrogen source, in contrast to the free amino acids. In this connection it may be recalled that a small quantity of extractable nitrogen did not dialyse, a result obtained by analyses of comparatively nitrogen-rich samples. According to Yemm and Folkes (1958), workers in the Botanical laboratory at the University of Bristol have shown that nitrogen-deficient barley is rich in peptides, a fraction which it was not attempted to determine in the present work. Mansford and Raper (1954) also accounted for around 90 per cent of the nitrogen in 75 per cent ethanol extracts of *Malus sylvestris* by amino acid analyses.

The amino acids of the ornithine cycle have lately been searched for in plant material by Kasting and Delwiche (1957), and found in young seed-

lings of wheat, barley and water-melon. As seen from figure 6, ornithine would reach a peak at tube 72 by the procedure employed. Ornithine was, however, never found in these extracts, even when the column was heavily loaded with extracted nitrogen. It seems at least safe to conclude that ornithine does not occur in the same quantity as it did in the plants analysed by Kasting and Delwiche (1 to 2 per cent of the arginine). Citrulline would emerge together with the acidic and neutral amino acids and the amides.

Traces of lysine were sometimes detected as a peak around tube 84.

3. *Accumulation of Nitrogenous Reserves*

The question of the building up of nitrogenous food reserve was not originally one of the problems to be dealt with in the present investigation. However, in the course of the work it became apparent that an understanding of the conditions determining storage accumulation would be of importance for the evaluation of the role possibly played by such reserves in the life of the trees. This problem can, however, only be discussed in a tentative way, because of the scarcity of pertinent experimental data.

External supply of nitrogen over a year never failed to produce plants with a store of nitrogenous reserves in the spring. But nitrogen application during the growing period did not always immediately result in accumulation of nitrogenous reserves. The different curves showing the changes in soluble and non-soluble nitrogen always demonstrate a decrease in soluble nitrogen during the growing season, and often a decrease in the non-soluble fraction as well. This decrease was found even if the plants were amply supplied with nitrogen. All the same the plants were found to contain large amounts of storage nitrogen early in the spring. The building up of reserves must therefore have taken place mainly during the season of dormancy.

Analyses of plant material taken during the autumn of 1955 from the Series I, 1955—56 experiment, demonstrate that accumulation may start late in the summer. The results also give an indication of the influence of the nutritional status of the plants on the accumulation process. Plant material was collected for analyses three times at intervals of 5 to 6 weeks from the 13th of August, when one group of previously nitrogen-starved plants was supplied with nitrogen according to the plan presented in Table 9. The results are presented in Figures 23 and 24.

External supply of nitrogen to plants which had not received nitrogen earlier in the season led to a very rapid rise in the amount of soluble nitrogen in both "stem" and roots. A much slighter and less progressive increase was found in the non-soluble fraction as a result of the nitrogen supply. In contrast to these plants stand those which had been supplied with nitrogen

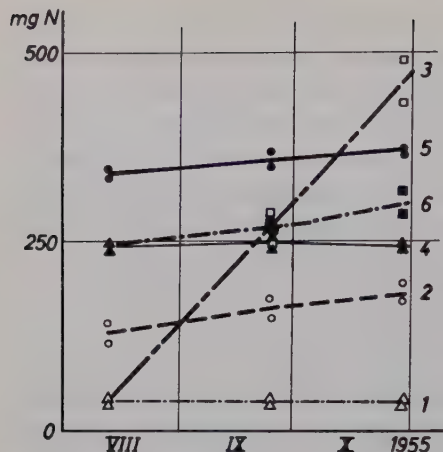


Figure 23. Soluble N (1, 2, and 3) and non-soluble N (4, 5, and 6) in stems of plants with different nitrogen treatment through a period with little growth. The N treatments: 1 and 4 without N throughout 1955; 2 and 5 with N throughout 1955; 3 and 6 as 1 and 4, but with nitrogen from the middle of August 1955. Coordinates as in Figure 11.

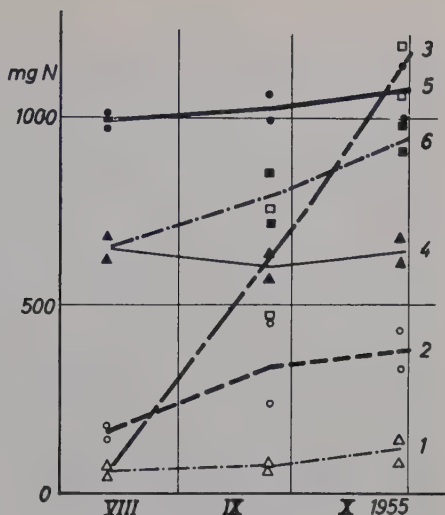
throughout the growing season (N/—). They showed only a rather inconspicuous increase in both nitrogen fractions over the same period.

The experiments do not furnish evidence for an explanation of these relations. However, the carbohydrate/nitrogen interaction in growth and metabolism may be assumed to play a decisive role. The plants which did not receive nitrogen until the 13th of August may have stored carbohydrate, which would be available for absorption of nitrogen and for rapid synthesis of organic nitrogen compounds. The plants which had been supplied with nitrogen throughout the season might in contrast have used their carbohydrate supplies for new growth.

In this connection should be recalled some of the results of Batjer *et al.* (1943) on the relation between nitrogen absorption and plant growth. During a period of approximately 3 weeks there appeared to be no nitrogen absorption and translocation in trees which received nitrogen after being previously grown without supply of nitrogen. Later the translocation was very rapid. Although these trees had been kept in culture without nitrogen for some time, they continued to increase in length until the high nitrogen treatment began. The terminal elongation ceased at the commencement of the high nitrogen treatment, probably due to concurrent chilling of the roots.

More difficult to explain is the high spring level of soluble nitrogen in the roots of plants which did not receive nitrogen the whole of the previous year. The actual observations are about 420 mg/100 g in both the Series I and Series II plants, and in table 18 is reported a soluble-nitrogen content of approximately 700 mg/100 g. A comparably high level of soluble nitrogen has not been found in the summer or autumn in any part of the plants grown without supply of nitrogen. The high quantity of soluble nitrogen cannot in

Figure 24. Soluble N (1, 2 and 3) and non-soluble N (4, 5 and 6) in roots of plants with different nitrogen treatment through a period with little growth. The N treatments: 1 and 4 without N throughout 1955; 2 and 5 with N throughout 1955; 3 and 6 as 1 and 4, but with nitrogen from the middle of August 1955. Coordinates as in Figure 11.



this case be explained by a coincident low level of non-soluble nitrogen in the same tissue. Thus, it must have been derived from other sources. It may be that young fibrous roots and root hairs have died during the winter, and that their nitrogen content has been absorbed by the living part of the roots. If this is the explanation, dieback of root cells or tissues must take place to quite a large extent during the winter. The high spring level of soluble nitrogen in the "stem" of the O— plants of Series II, *viz.*, 210 mg/100 g, may be explained as due to nitrogen reabsorbed from the leaves in the autumn.

VII. Discussion

An initial extraction of apple tree tissue by 70 per cent aqueous ethanol cannot be considered satisfactory when the aim is to obtain most of the free amino acids in solution. With material of *Claviceps litoralis*, Berman (1957) found the boiling water method to be more efficient in extracting amino acids than 70 per cent alcohol or cold 5 per cent trichloroacetic acid. Steward and Street (1946) found indications that aqueous extracts of potato tubers contain substances, probably basic, which are absent from alcoholic extracts. Working with material of a series of plants and plant parts, Stuart (1935) found that without exception extraction with water removed larger amounts of nitrogen than did alcohol. This was reflected in higher concentrations of all the soluble nitrogen fractions with the exception of ammonia. The difference was usually most pronounced in the basic fraction. These results

indicate that the free amino acids of other biological material may also be incompletely extracted by 70 per cent ethanol. The "probably basic substances" in the article by Steward and Street (1946) and the "basic fraction" in the paper of Stuart (1935) are probably mainly arginine, the amino acid which in the present work has been shown to be particularly difficult to remove by means of aqueous ethanol.

A letter indicating some preliminary results on the extraction of amino acids from apple tree tissue was published by Oland and Yemm (1956). The results have been commented on by Steward and Pollard (1957) and Yemm and Folkes (1958). Steward and Pollard suggest that the procedure used on the ion exchange column might not be sufficiently accurate for the determination of asparagine separate from glutamine. However, the overall picture given by the letter would appear to be consistent irrespective of the validity of the criticism offered on this special point. Yemm and Folkes find that the inability of aqueous ethanol to remove arginine may not be sufficiently realized. They give examples where the extraction procedure may be suspected of having introduced errors.

Apple stem material adsorbs arginine from aqueous ethanol or cold aqueous solutions, but not from a weak salt solution. A similar adsorption of glutamic acid may occur, but to a lesser degree. This adsorption may be associated with the presence of weak acidic groups in the plant material, and may, further, offer an explanation of why the free amino acids are difficult to extract with 70 per cent ethanol.

Indications were found that qualitative changes may take place in the free amino acids when they are extracted by 70 per cent ethanol and the extract concentrated in vacuo at 40°C. In this connection the results of Webster (1933) should be recalled. He found that progressive changes took place in the nitrogenous fractions when plant material was stored in alcohol. A continually decreasing percentage of amino nitrogen was found, and conversely an increasing percentage of ammonia. Generally the decrease in amino nitrogen was greater than could be accounted for by the increase in ammonia. The results of Webster were confirmed by Stuart (1935). Thus, there may be a danger of a general occurrence of qualitative changes in the amino acid fraction when plant material is treated with ethanol. It is probably premature to speculate on the types of changes which may occur. The presented data should, however, be sufficiently clear to call for care in the use of ethanol in quantitative studies of the free amino acids in plant material.

There will probably always be some uncertainty as to what is obtained by an initial extraction of plant material, whatever extractant and extraction procedure is used. It seems, however, satisfactory when different methods yield results in close agreement, as has been shown in the present study.

That a satisfactory extraction has been obtained is indicated by the fact that little non-dialysable nitrogen was extracted, and also by the high percentage of the soluble nitrogen accounted for in low-molecular weight compounds.

The preparation and preservation of plant material prior to analysis may also give rise to changes in the nitrogenous compounds. It seems, however, to be well established that the same amount of soluble nitrogen is obtained whether the material is extracted fresh, frozen or dried. This is in agreement with the findings of Lincoln and Mulay (1929). In experiments with apple tissues they found that the same amount of nitrogen was extracted from material dehydrated at 50 to 55°C, material stored for a short period at 0°, or frozen for a longer period, as from fresh material. It is common to dry plant material for nitrogen analysis at 70—80°C (Nightingale 1937). This should, however, not be done when quantitative determinations of the single free amino acids are wanted. In view of recently gained knowledge on reactions between carbohydrates and amino acids (Synge 1955), it is likely that drying may accelerate such reactions and consequently lead to errors in the determinations of amino nitrogen. The magnitude of the error introduced would be difficult to assess because variables, such as moisture content and temperature, influence the reaction.

Quantities of individual compounds or small groups of compounds, obtained by analyses of extracts on the ion exchange column, are given as nitrogen content in per cent of the total soluble nitrogen. The results are then available for critical use, in contrast to the situation when quantities are given as per cent of the total nitrogen accounted for (cf. Steward *et al.* 1958).

On the whole, the chemical methods employed seem adequate for the purpose of the present investigation.

It is obvious that the free amino acids are important as nitrogenous reserves in apple trees. Quantitative changes in the pool of these amino acids occur when new growth makes a demand on reserve nitrogen, or under conditions favourable for an increase in the nitrogenous reserves. In the apple tree the nitrogen of the free amino acids (the amides included) appears to be practically equal to the nitrogen extracted by weak salt solutions, hot water or diluted acids. An exception from this has to be made for plants exhausted of soluble nitrogen.

Only a few compounds in the soluble fraction, arginine in particular, and also the amides, show the behaviour of typical storage compounds. Asparagine is probably the dominating amide, according to Oland and Yemm (1956) and Oland (1954). It may be of interest to note that Oland (1954) found comparatively more glutamine in the maidens than in the roots of young apple plants, and that Bollard (1953) found glutamine to be a predominant nitrogenous compound of the tracheal sap of one-year shoots of mature apple

trees. These findings would indicate that the transported compounds may be other than the storage compounds.

As mentioned in the introduction, it has been presumed by several investigators that the free amino acids are storage compounds. An easy way to determine the main compounds has been to analyse the storage organs of collected plants, as extensively done by Reuter (1957). The characteristic of the typical storage compound is a great fluctuation in concentration. Therefore, it is obvious that the plants should be known to be rich in nitrogen in general at the time of analysis before the main compound can be assessed. From the present results it can be concluded that the apple tree would not give the right picture with respect to main storage compound if analysed in the growing season and with low supply of nitrogen. However, a vast number of analyses in the work of Reuter (1957), and the systematic appearance of some compounds, make it clear that low-molecular weight compounds other than arginine and the amides may serve as storage compounds in plants.

Besides the free amino acids, a part of the non-soluble fraction behaves as a nitrogenous reserve. This non-soluble nitrogen is assumed to be protein. However, apart from the indications that free amino acids have been practically completely extracted, there is no direct evidence in the present work of all the non-soluble nitrogen being proteinaceous in nature.

Virtanen (1952) has advanced the hypothesis that all the protein in young active cells is enzyme protein. It may be assumed that whether the protein acts in a typical protein function, as for instance in enzymes, or is drawn upon as a nitrogenous reserve, depends on the supply of nitrogen to the organism. In the present work there is evidence both in favour of and against this hypothesis. The downward trend in non-soluble nitrogen parallel to the decrease in the soluble fraction in the roots of plants with an initial high quantity of total nitrogen, is in agreement with the examples given by Virtanen.

On the other hand, the stem wood of plants rich in nitrogen shows a rapid decrease in non-soluble nitrogen to a level very near to that of nitrogen-poor plants. This happens in the growing season and even if the plants are externally supplied with nitrogen. If the reserve protein acts as constitutional protein at all, it would be expected to do so in the growing season, and would not be expected to be re-distributed when the plants are externally supplied with nitrogen. It seems likely, therefore, that at least the wood may contain true reserve protein. If so, it might be possible to isolate this protein fraction, in the same way as Danielson (1954) has isolated particular proteins of pea-seeds and shown them to act as nitrogenous reserves during germination and early growth.

The components which can be assumed from the present experiment to be nitrogenous reserves show a decrease throughout the growing season, irrespective of nitrogen treatment of the plants. This indicates that the trees do not economize particularly with the nitrogen of the reserves. The utilization in the growing season, even with plentiful external supply of nitrogen, must lead to the conclusion that the nitrogenous reserves have a main function in a short-time saving program.

Plants which once have reached a high nitrogen level do, however, show a "more than one step decrease" in the concentration when grown without nitrogen supply for more than one growth season. The storage organs then show a nitrogen increase in the intermediary dormancy season. It seems probable that the very high levels of storage nitrogen are reached by absorption of nitrogen, immediate synthesis, and accumulation of nitrogenous reserves. The first concentration-decreasing step is the occurrence of new growth by utilization of the free amino acids and true reserve protein. The increase in nitrogen concentration, particularly in free amino acids, of the storage organs in the intermediary dormancy season is probably due to reabsorption of nitrogen from old leaves before abscission and from dying root-hairs. This nitrogen can be drawn on by the next seasons growth.

The accumulation of the reserves has often been found to take place during the season of dormancy, and further, the probable dependence on carbohydrate reserves has been mentioned. Thus it may be assumed that the nitrogenous reserves may often be built on the late season's photosynthesis products.

In practical orcharding, the conditions for building of nitrogenous reserves during the season of dormancy may vary considerably, and thus also the quantity of reserves in the spring. Under the conditions of a continental climate, frost usually occurs soon after the late apple varieties have been harvested. There will be little time for post-harvest photosynthesis. The temperature may also be below the working minimum for the nitrogen assimilation process. An oceanic climate may, on the other hand, frequently have long postharvest periods with temperatures above the minimum for the building up of carbohydrate reserves, and the winter temperature may be suitable for nitrogen assimilation. Thus, the importance of nitrogenous reserves may vary according to climatic conditions. This will again give possibilities for varying responses to nitrogen fertilization.

The underlining of the importance of the dormancy season for the building up of nitrogenous reserves is in agreement with the results of Murneek (1942). Although he stresses the downward transport of carbohydrates and nitrogenous compounds reabsorbed from the leaves as being the important process in the autumn, there are strong indications in his work that absorp-

tion of nitrogen from the soil do take place. Whether the absorption is due to the late season's photosynthesis products or to the autumnal downward transport of carbohydrate can only be settled by further experiments.

It may be a general feature that vigorous nitrogen assimilation is dependent, not only on available carbohydrate, but on rather large carbohydrate reserves. Willis and Yemm (1955) found indications in barley plants that a condition of nitrogen deficiency together with a high content of carbohydrate are features leading to greatest respiratory increase. And the experiments suggest that it is the metabolic changes associated with the synthesis of nitrogenous constituents in the roots which lead to high rates of carbohydrate utilization and an increase of cellular respiration. Syrett (1956), working with *Chlorella*, found that nitrogen assimilation was particularly dependent on large carbohydrate reserves when nitrate was the nitrogen source. With *Torulopsis utilis*, Yemm and Folkes (1954) obtained results which clearly indicate that a high reserve of carbohydrate together with a low level of soluble nitrogen are two important factors influencing the assimilation of nitrogen. The results in the present work of one single experiment on the building up of nitrogenous reserves would indicate that these factors are also important for vigorous absorption of nitrogen by apple trees. It must be added that these two factors did not occur independently, as nitrogen starvation was the immediate cause supposed to lead to storage of carbohydrate. Under orchard conditions, storage of carbohydrate may be due to other causes than stunted growth because of nitrogen starvation. It can probably be safely assumed that the accidental absence of crop for one or several years would lead to storage of carbohydrate in the trees. Such conditions may frequently occur and result in absorption of available nitrogen and accumulation of nitrogenous reserve food. To what extent accumulation of nitrogenous reserves actually takes place in such situations can only be assessed by adequate experiments with trees of fruiting age.

The young apple plants of the present experiments have been shown to be able to grow vigorously on the nitrogenous reserve food. Such plants may increase their original weight three or four times without external supply of nitrogen. But it is obviously difficult from these results to evaluate the importance of the nitrogenous reserves for the production of older trees.

There are no indications in the present work that upward transport may be a limiting factor in the utilization of the nitrogenous reserves of the stems and roots. The view maintained by Miller (1938), and in part by Curtis and Clark (1950), that there is little upward transport of reserve food, may probably refer to experiments with trees having small amounts of reserves, due to, *e.g.*, low supply of nitrogen. The same limitation may be expected for the observations of Curtis (1920) that new growth only draws upon twigs and

medium-sized branches for stored food. Loomis (1935) seems to distinguish between old and young trees with respect to from where the new growth draws reserve food. He states that because young trees have a relatively small aerial space, the reserves of the trunk and the roots may also be used. It is difficult to judge from available information to what extent older trees may have translocation problems with respect to the utilization of the nitrogenous reserves of the roots and the stem.

The conclusions of the present work seem to suggest that a study of the accumulation of nitrogenous reserve food under conditions of ample carbohydrate reserves would lead to a better understanding of the role played by nitrogenous storage compounds in fruit trees. Experiments should be carried out with trees of fruiting age. A study of the normal concentrations of nitrogenous reserves obtained in trees under different climatic conditions might also lead to a better understanding of nitrogen fertilization problems. At least the free amino acid pool can at present be determined by chemical methods.

VIII. Summary

1) This investigation deals with methods for the determination of nitrogenous fractions and compounds in material of young apple trees; with the significance of nitrogenous reserves for new growth; with the accumulation of reserves; and with the nature of the nitrogenous reserve compounds.

2) A considerable gain of time was achieved by changing the resin of the 15 cm ion exchange column from one of 8 to one of 12 per cent cross-linkage. Small changes in the buffers from those used by Moore and Stein have also to be made. The modified procedure was more than four times faster than the original one.

3) Amide nitrogen can be determined by distillation with saturated NaOH in the Parnass-Wagener apparatus, mainly as described by Varner *et al.* The main alteration needed consisted in separating the arginine from the amides on an ion exchange column prior to the determination of the amide nitrogen.

4) Seventy per cent aqueous ethanol does not extract all free amino acids of the apple tree tissues. Cumulative extraction curves indicated practically complete extraction after the use of 500 ml of extractant to a few grammes of plant material. Nevertheless, subsequent extraction of the same plant material by other extractants removed comparatively large amounts of nitrogenous compounds, particularly arginine.

5) Apple stem material adsorbs arginine from aqueous ethanol or cold aqueous solutions, but not when it is dissolved in a weak salt solution. A similar adsorption of glutamic acid may also occur, but to a lesser degree.

6) Qualitative changes in the nitrogenous compounds extracted by 70 per cent ethanol take place during extraction and/or concentration of the extract previous to analysis on the ion exchange column.

7) Different extractants, such as hot water, weak salt solutions and diluted hydrochloric acid, and different extraction procedures, extract the same total quantity of nitrogen from apple tree tissues. For routine use the soluble nitrogen was extracted with 0.2 M NaCl buffered by phosphate to pH 7. Practically all the nitrogen obtained by this extractant dialyses against water. Nitrogenous compounds are not precipitated from the extract by trichloroacetic acid.

8) The free amino acids of apple tree tissues can apparently be extracted completely by percolation with the same buffer (pH 4) as is needed to put them on to the ion exchange column, and with a concentration of the extract which is suitable for direct analysis. Up to 3.5 grammes of plant material have been extracted with only 10 ml of buffer.

9) The same quantity of soluble nitrogen was extracted whether the material was fresh, frozen or dried. The amount of amino nitrogen was, however, significantly lower in the extracts of dried material as compared to those of fresh and frozen material. Qualitative changes in the nitrogenous compounds may take place during the drying of the plant material.

10) The nitrogen treatments of young apple trees during one year show marked effects the following year. The effects of the previous treatments may be detected even under the influence of widely different current nitrogen treatments. Some of the main effects have been brought about through the mobilization of nitrogenous reserves.

11) The current year's growth is mainly a function of the initial level of nitrogenous reserves and of current supply of nitrogen. The stored nitrogen seems always to be used for new growth, irrespective of current supply. Therefore, if the plants contain large amounts of storage nitrogen, the effect on the growth of the current supply may be very small and only noticeably at the end of the growing season.

12) There is a close exponential relationship between both fresh and dry weight and the height of the new shoots (maidens).

13) The soluble nitrogen, largely free amino acids, has a main function as a nitrogenous reserve. The concentration of this fraction in tissues suitable for storage was highest at the time of initiation of growth, and lowest at the end of the growing season.

14) Of the free amino acids arginine was the most typical and quantitatively most important storage compound. It is found in large quantities in storage tissues rich in nitrogen, whereas the same tissues may be extremely depleted of arginine after new growth has occurred without external supply

of nitrogen. The amides also show the character of storage compounds, but nitrogen-starved tissues have never been found depleted of amides to the same extent as of arginine. From 80 to 90 per cent of the soluble nitrogen of plants rich in nitrogen has been accounted for as arginine and amides.

15) A part of the non-soluble nitrogen serves as reserve food. Prior to initiation of growth there is a decrease in the non-soluble nitrogen accompanied by an increase in the soluble fraction. This phenomenon has been interpreted as a mobilization of the non-soluble nitrogenous reserves. Of plants which have built up large reserves, the roots seem to be the part with the relatively highest level of non-soluble reserve nitrogen.

16) In the leaf tissue the concentration of soluble nitrogen is practically constant, and low, throughout the season. High concentrations of the free amino acids are thus only found in tissues suitable for storage.

17) Not all the effects of the previous nitrogen treatment on the current growth and nitrogen constitution of the plants can be explained by a simple operation of nitrogenous reserve compounds.

18) From analyses during the growing season, it is concluded that accumulation of nitrogenous reserves in plants with a constant, high supply of nitrogen occurs from late autumn till spring. Previously nitrogen-starved plants may accumulate reserves in the summer and early autumn if supplied with nitrogen. These findings are taken to indicate a competition for carbohydrate between growth processes and synthesis of nitrogenous reserves; the growth processes seems to have priority in this competition.

19) Plants which were supplied with nitrogen during the previous season terminated growth earlier in the season than plants with no such supply, irrespective of current supply. Plants terminating growth early were found to have a low content of soluble nitrogen in the storage tissues and also a low content of total nitrogen in the leaves. The early termination of growth even with ample supplies of nitrogen may be due to inhibition of nitrogen absorption and assimilation, caused by carbohydrate shortage after the vigorous growth over two seasons.

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Chemical Analyses of Microsomal Ribonucleoproteins and Ribonucleic Acids from Germinating Bean Seeds

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With the advancement of the germination stage an orderly change has been found to take place in the electrophoretic patterns of the pH 7.1-fractions of bean seed tissues. Thus in good accordance with our view on the interconvertible two existing forms of cytoplasmic ribonucleic acids, *i.e.*, functional and transportable RNA-s, a conversion of Component II of a moderate mobility (assumably ribonucleoprotein that is to be designated as functional RNA) into Components III and further IV of higher mobilities (probably free RNA-s antecedent to transportable RNA) was suggested to occur in the aged microsomes (Oota and Takata). The electrophoretic components have now been isolated and their protein and RNA contents, their ultraviolet absorption spectra and nucleotide compositions of their nucleic acid fractions were determined. As described below, the results obtained not only verify our postulation on the chemical property of each component substance of the pH 7.1-fractions but permit of some further speculation on the mode of the assumptive RNA interconversion.

Materials and Methods

The seeds of *Vigna sesquipedalis* stored in a dark desiccator for about two and a half years were used. Procedures of seed germination, preparation of pH 7.1-fraction and electrophoresis were detailed previously (Oota and Takata). This time a Spinco Model H electrophoresis-diffusion apparatus was used in place of a Hitachi Model HT-B apparatus.

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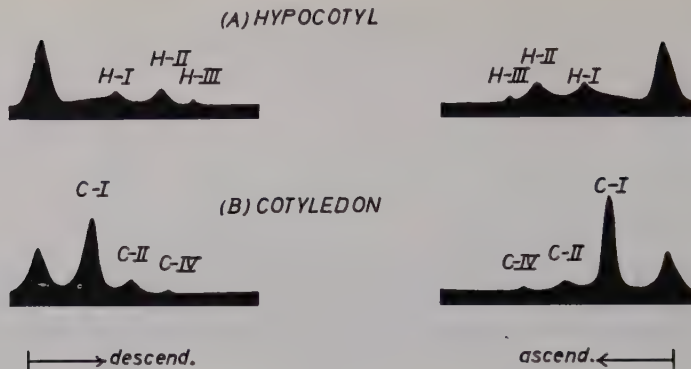


Figure 1. *Electrophoresis diagrams of pH 7.1-fractions of 1 day-old bean germ tissues.* (a) Hypocotyl preparation. (b) Cotyledon preparation. Spinco Model H apparatus; 11 ml cell. Phosphate buffer, pH 7.1, 0.1 *M*; 2°C.; field strength, 4.4 volts/cm; (a) 114 min. and (b) 100 min. Calculated descending mobilities (*u*): -6.8 (H-I), -10.2 (H-II), -12.6 (H-III), -5.6 (C-I), -8.9 (C-II) and -13.5 (C-IV).

Isolation of electrophoretic components. From ca. 1,000 hypocotyls and ca. 300 pairs of cotyledons of 1 day-old germs, 25 ml of the pH 7.1-fractions were prepared, respectively. The fraction from either organ has been reported to yield three distinct electrophoretic components, namely H-I (mobility, $u = -6$), H-II ($u = -10$) and H-III ($u = -12$) for the hypocotyls and C-I ($u = -6$), C-II ($u = -10$) and C-IV ($u = -14$) for the cotyledons (Oota and Takata). As seen in Figure 1, it was also true of the present samples excepting that by some obscure reason the cotyledon pH 7.1-fractions gave C-II of a smaller mobility of -9. Two electrophoretic runs, each with a 11-ml cell, were conducted in parallel at 2°C. and a field strength of 4.4 volts/cm. Under the present conditions migration of ca 2 hr. duration was found suitable to the isolation of the components. After the bottom section of the cell assembly was displaced so as to prevent disturbance in formed boundaries, the fastest moving component (H-III or C-IV) as water-clear liquid and the slowest moving (H-I or C-I) as rather turbid liquid were taken in limited amounts by means of a sampler attachment out of the ascending and the descending limbs, respectively. Finally the turbid portion of the residual solution in the whole cell was drawn out thoroughly by the syringe and designated as *Mix*, which was apparently constituted of the bulk of the leading and the trailing components as well as the whole of the component of intermediate mobility (H-II or C-II). On the other hand, from separate runs the fastest moving two components, *i.e.*, H-II and -III or C-II and -IV, were pipetted out together of the ascending limbs in a similar way to the case of isolation of H-III or C-IV. The combined components were nearly water-clear and named H-II-III or C-II-IV.

Ultraviolet spectroscopy. Aliquots of the isolated components or component mixtures, after adequate dilution with a phosphate buffer (pH 7.1, 0.1 *M*) if needed, were immediately used for the determination of their ultraviolet absorption spectra. The remaining portions of the samples were stored in a deep-freezer at ca. -20°C. The optical density measurements were conducted by a Beckman Model DU spectrophotometer throughout the present study.

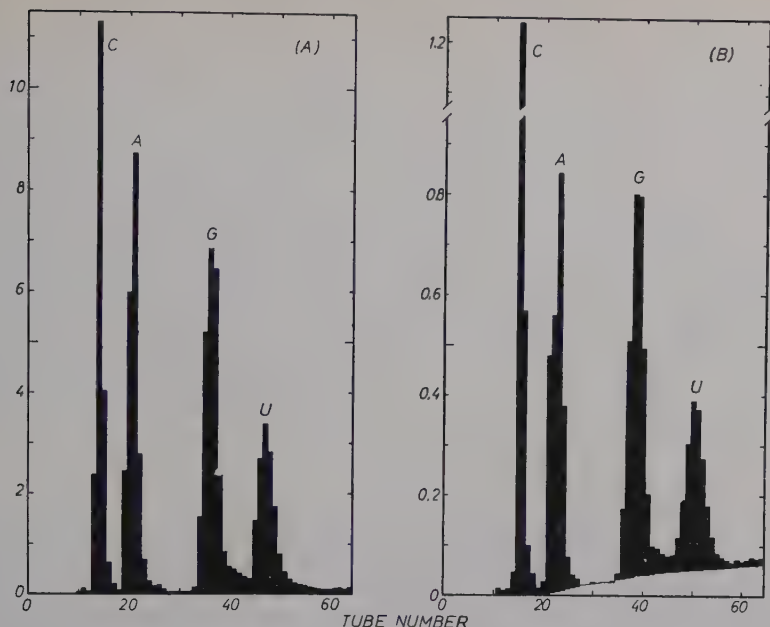


Figure 2. Nucleotide patterns of RNA-s from electrophoretic components of pH 7.1-fractions of 1 day-old bean germ tissues. Exchanger: Dowex-1 ($\times 2$, 200—400 mesh)-formate, $0.3 \text{ cm}^3 \times 20 \text{ cm}$. Sorbed materials: (a) Digest (0.5 N KOH , 37°C ., 18 hrs) of RNA powder from H-Mix (total $E_{260}=319$). (b) Digest of RNA powder from C-IV (total $E_{260}=34.8$). Eluting solution: 30 ml of 1 N , followed by 200 ml of 4 N formic acid. Recovery of elution as estimated as nucleotide E_{260} : (a) 90 per cent, (b) 97 per cent. *Abscissa*: Number of fractionation tubes, each containing 4 ml of eluate. Blank area in the diagram (b) indicates the absorbancy due to the eluting solution used. *Ordinate*: Optical density per ml at $280 \text{ m}\mu$ for cytidylic acid (C) and at $260 \text{ m}\mu$ for adenylic (A), guanylic (G) and uridylic (U) acids.

Nucleotide analysis. The above component isolation was repeated several times on separate preparations of pH 7.1-fractions in a 10 to 20 days period until sufficient amounts of minor components (H-I, H-III and C-IV) were obtained. It has been found that the pH 7.1-fractions or *Mix* can be stored at -20° for at least 20 days without any detectable change in their nucleotide compositions.

The anion exchange method essentially as given by Osawa *et al.* was applied in the separation of nucleotides. From the samples acid- and alcohol/ether-soluble substances were removed at 0° . The resulting crude RNA powder was wholly digested with a small amount of 0.5 N KOH (37° , 18 hrs), and centrifuged. The supernatant was neutralized up to ca. pH 8 with 3 N HClO_4 , clarified by centrifugation, and, after optical density at $260 \text{ m}\mu$ was estimated, adsorbed to a Dowex-1 ($\times 2$, 200—400 mesh)-formate column ($0.3 \text{ cm}^2 \times 20 \text{ cm}$). Gradient elution was carried out first with 30 ml of 1 N formic acid and subsequently with 200 ml of 4 N formic acid. Optical densities per ml at $260 \text{ m}\mu$ (E_{260}) and $280 \text{ m}\mu$ (E_{280}) were read for every 4 ml eluate. The obtained elution patterns (cf. Figure 2) fairly agreed with that of

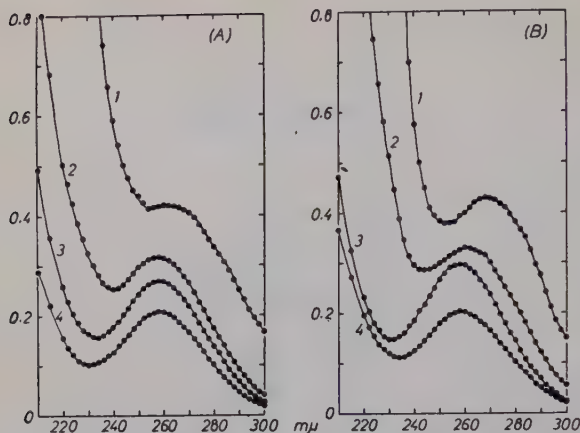


Figure 3. Ultraviolet absorption spectra of electrophoretic components isolated from pH 7.1-fractions of 1 day-old bean germ tissues. (A) Hypocotyl preparations. Curve 1, H-I; Curve 2, H-Mix; Curve 3, H-II-III and Curve 4, H-III. (B) Cotyledon preparations. Curve 1, C-I; Curve 2, C-Mix; Curve 3, C-IV and Curve 4, C-II-IV. Medium: Phosphate buffer, pH 7.1, 0.1 M. Abscissa: Wave length in $m\mu$. Ordinate: Optical density.

authentic yeast RNA. Taking arbitrarily the adenylic acid content (in moles) as 10, relative molar contents of the separated nucleotides were calculated by the following equations: guanylic acid = $\text{total } E_{260} \text{ for guanylic peak} / \text{total } E_{260} \text{ for adenylic peak} \times 12.0$, cytidylic acid = $\text{total } E_{280} \text{ for cytidylic peak} / \text{total } E_{260} \text{ for adenylic peak} \times 10.9$, and uridylic acid = $\text{total } E_{260} \text{ for uridylic peak} / \text{total } E_{260} \text{ for adenylic peak} \times 14.4$. Total optical density of a given nucleotide was assayed by computing the area under the peak of the nucleotide under consideration in the elution diagram. When the samples of smaller total E_{260} were examined, the separately determined blank absorbancy by the eluting solution (formic acid) was to be subtracted (cf. Figure 2-b).

Protein estimation. This was made by the method of Lowry *et al.* (cf. Layne). To an aliquot of the sample was added 5 vol. of the alkaline copper solution, and it was followed, 10 min. later, by the addition of $1/2$ vol. of the Folin-Ciocalteu reagent. After the mixture was left for 30 min. at room temperature, the optical density was read at 500 $m\mu$. Crystalline human serum albumin was used as protein standard.

Nucleic acid estimation. The RNA contents were estimated from total E_{260} determined above for the alkali digests. The conversion factor, 33 μg RNA per E_{260} , which had been preliminarily obtained for yeast RNA was adopted.

Unless otherwise stated, all the analyses were repeated more than twice, and the mean values are given below.

Results and Discussion

Absorption at ultraviolet region of isolated components are shown in Figure 3. Either of the most mobile H-III and C-IV gave a typical RNA spectrum which exhibits an absorption maximum at 258 $m\mu$ and a minimum at 230 $m\mu$; the ratio E_{258}/E_{230} amounted to as high as slightly more than 2. The least mobile components (H-I and C-I) gave the absorption curves markedly smoothed and with the cardinal points shifted considerably toward

Table 1. *Protein and RNA contents of electrophoretic components isolated from pH 7.1-fractions of bean hypocotyls and cotyledons.* Contents per 100 hypocotyls and 50 pairs of cotyledons shown. Protein was assayed colorimetrically by the Folin method using human serum albumin as a standard. RNA was estimated indirectly from E_{260} of alkali digest of crude RNA preparation, the conversion factor adopted was 33. For other details, see the text.

Tissues	Hypocotyl				Cotyledon			
Components	H-I	H-Mix	H-II-III	H-III	C-I	C-Mix	C-II-IV	C-IV
Protein (μ g) ...	298	5,620	120	0	2,717	25,400	323	0
RNA (μ g)	37. 6	2,445	160	11.1	142	3,260	338	26.5
RNA Protein	0.13	0.43	1.33	∞	0.05	0.13	1.05	∞

longer wave-length; much or less definite absorption maximum at 280 m μ (protein peak) being detected. The curves for the component mixtures were of intermediate shape of the two extremes just mentioned.

Table 1 shows the protein and RNA contents of respective components. In agreement with what the above spectra indicated, the fastest moving components gave perfectly negative Folin reaction. In view of their high mobilities comparable to those of H-III and C-IV, C-III ($u = -12$) and H-IV ($u = -14$) may also be ascribed to free RNA-s. As anticipated, an obvious parallelism was revealed between the RNA concentration on a protein basis and the electrophoretic mobility of the component. It is, however, hardly possible to show in this place exact factor(s) on which the mobility of free RNA (H-III, C-IV, also probably H-IV and C-III) depends; increase in electric charge and/or decrease in molecular size can affect the mobility of RNA molecule.

The RNA/protein ratios of both H-II-III and C-II-IV slightly exceeded unity (cf. Table 1). Considering small amounts of free RNA, *i.e.*, H-III or C-IV, in these samples, it is nearly explicit that the components of intermediate mobility (H-II and C-II) may be typical ribonucleoproteins consisting of equal amounts of RNA and protein. According to our hypothesis, H-II or C-II is the essential microsomal ribonucleoprotein which participates or has participated in the synthesis of protein (Oota and Takata).

H-I and C-I gave the lowest RNA concentrations, and, moreover, it was our experience that the determination of RNA contents of these least mobile components yielded little reproducible results. This will readily be explained from our view that the components in question are probably best visualized as membranous fragments attached by RNA (Oota and Takata). Phosphatid analysis was not made for the isolated components.

The nucleotids patterns determined for the digests of nucleic acids from the present materials wer \acute{e} , as noted earlier, in a fair agreement with that

Table 2. Nucleotide compositions of RNA-s from electrophoretic components of pH 7.1-fractions of bean hypocotyls and cotyledons. Molar ratios (adenylic acid=10.0) indicated.

For experimental details, see the explanation of Figure 2 and the text.

Tissues	Hypocotyls						Cotyledons					
Components	pH 7.1-fraction	H-I ¹	H-Mix	H-II-III	H-III ¹	mean	pH 7.1-fraction	C-I	C-Mix	C-II-IV	C-IV ¹	mean
Guanylic ...	14.1	14.3	14.1	14.5	15.8	14.6	14.2	14.5	14.1	13.9	15.0	14.3
Cytidylic ...	9.8	9.0	9.9	10.0	10.7	9.7	10.1	10.9	10.3	9.9	10.7	10.4
Uridylic ...	10.7	9.7	10.4	10.3	10.6	10.3	10.4	11.2	10.4	10.7	11.0	10.7
Purines												
Pyrimidines	1.17	1.30	1.19	1.21	1.21	1.23	1.18	1.11	1.16	1.16	1.15	1.15

¹ Estimation conducted only once.

separately ascertained for the digest of authentic yeast RNA. Representative elution diagrams are given in Figure 2. The absence of thymine peak reflected freedom of the preparations from deoxyribonucleic acid. Interesting enough a striking coincidence was found in the molar proportion of nucleotides for all the RNA preparations studied. Thus the ratios, adenylic/guanylic/cytidylic/uridylic, were approximately 10.0/14.5/10.1/10.5 irrespective of the kinds of not only the components from which the RNA-s were derived but the tissues from which the components were isolated (Table 2). We might here be allowed to conclude that all the components including those not studied here, *i.e.*, H-IV and C-III, may contain RNA of the same nucleotide composition. If so, the interconversion of the components as assumed in our hypothesis, *i.e.*, II→III→IV, should not involve any modification in the nucleotide composition of the RNA moiety.

Lindner *et al.* were likely the first in separating free RNA particles from plant cytoplasm. Thus the microsomes of immature leaves of several plant species were demonstrated electrophoretically to contain a few kinds of free RNA-s. For example, pH 7.0 buffer (composed of 0.02 *M* neutral potassium phosphate and 0.15 *M* potassium chloride) extract of the microsome (collected in a gravity range of 20,000—145,000×*g*) from cowpea (*Vigna sinensis*) leaves yielded three RNA peaks, *i.e.*, N₁ (*u*=−7.4), N₂ (*u*=−8.4) and N₄ (*u*=−15), besides three ribonucleoprotein peaks, *i.e.*, P₁ (*u*=−0.7), P₂ (*u*=−3.5) and P₃ (*u*=−6.0). Although these authors reported negative biuret and ninhydrin reactions for the combined free RNA-s (N₁+N₂+N₄), it appears dubious if N₁ and N₂ of such a low respective mobilities as −7 and −8 can really be free RNA-s. The descending mobilities of free RNA-s are known to lie usually around −13 (*cf.* Watanabe and Suzuki). That the most mobile N₄ with its mobility of −15 will be free RNA is acceptable. An implication, however, of their results that the free RNA-s would serve as a reserve is interesting in connexion with our view on the role of free RNA as transportable RNA. They

Table 3. A comparison of nucleotids compositions of RNA-s from various plant sources (Molar relationships : adenylic acid=10.0). Data cited were recalculated on an adenylic acid basis.

Plants	Guanylic acid	Cytidylic acid	Uridylic acid	Purines	Authors
				Pyrimidines	
Soybean (meal).....	13.4	9.2	7.4	1.41	Di Carlo <i>et al.</i>
Soybean (leaves).....	15.0	10.9	9.6	1.22	Lindner <i>et al.</i>
Tobacco (leaves).....	14.3	10.0	8.3	1.33	Reddi
Tobacco (leaves).....	13.8	10.4	7.9	1.30	Lindner <i>et al.</i>
Wheat (germs).....	13.1	12.3	9.3	1.07	Lipshitz and Chargaff
Fern (prothallium)...	12.6	9.2	10.1	1.17	Hotta <i>et al.</i>
Bean (germs).....	14.5	10.1	10.5	1.19	Present results

have pointed out that their free RNA is apparently a common constituent of young tissues, and moreover a small amount of free RNA contained in the cotyledons of cucumber seeds diminishes rapidly with the initiation of germination. Removal of the epicotyl and axillary buds was found to result in a considerable accumulation of free RNA in the storehouse tissues in question.

Lindner *et al.* have also demonstrated a significant difference, as they stated so, in molar relationship of purine and pyrimidine bases between their free and bound RNA-s. For example, in bean (*Phaseolus vulgaris*) leaves, for free RNA-s (N_1 , N_2 and N_4 combined) the ratio, adenine/guanine/cytosine/uracil, is about 10.0/15.0/13.5/11.0, while for RNA from the whole extract ($N_1 + N_2 + N_4 + P_1 + P_2 + P_3$) 10.0/15.0/10.9/9.6 (the original data were recalculated on an adenine basis). If their free RNA may be identical with our transportable RNA, a serious reconstruction of free RNA molecules has to occur before they are converted into what we call functional RNA. This is, however, likely hardly consistent with our previous findings in the Vigna seed germs, which have led us to an assumption of a prompt conversion of transportable RNA into functional without accompanying any severe modification in the molecules. Thus on the one hand, at the outset of germination the decrease in the RNA content of a pair of cotyledons is apparently strictly recovered as the increase in the RNA level of the seedling axis (Oota and Osawa), and on the other, at the latter half of the germination stage a similar relation holds true between the so-called secondary storehouse tissues, that is the hypocotyls, and the epicotyls (Oota and Osawa, unpublished; cf. Oota).

By the way, the impression we get from the rather meagre informations so far available is that plant RNA-s are characterized by a high guanylic acid content and a low and roughly equal contents of adenylic, cytidylic and uridylic acids (cf. Table 3).

Summary

Electrophoretic components of the pH 7.1-fractions from 1 day-old bean hypocotyls and cotyledons, *i.e.*, H-I, -III, -II-III and -Mix and C-I, -IV, -II-IV and -Mix, were isolated, and their chemical properties were analysed. The most mobile and minor components, *i.e.*, H-III ($u = -12$) and C-IV ($u = -14$), yielded negative Folin reaction and ultraviolet absorption spectra characteristic to free RNA. H-II-III and C-II-IV gave the RNA/protein ratios close to unity, suggesting that the components of intermediate mobility ($u = -10$), *i.e.*, H-II and C-II, may be composed of equal amounts of RNA and protein. The least mobile H-I and C-I ($u = -6$) yielded the smallest and scarcely reproducible RNA/protein ratios. Homogeneity of RNA derived from every component or component mixture from every tissue examined was revealed with respect to the nucleotide composition: the common molar ratio, adenylic/guanylic/cytidylic/uridylic, being nearly 10.0/14.5/10.1/10.5. The findings were likely in favor of our hypothesis on functional and transportable RNAs in bean germ tissues.

We have profited from discussion with Dr. S. Osawa of this institute.

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Radioactive Iodine Incorporation into Organic Compounds of Various Angiosperms

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Introduction

An extensive early literature describes the concentration of iodine by marine algae, particularly the large brown seaweeds, and the manner of iodine combination in proteins of these plants. A survey of this work is available (Blinks 1951). Recent work has extended these earlier observations, and now there is evidence for the existence of mono- and di-iodotyrosine and di- and tri-iodothyronine in certain seaweeds. Roche and Yagi (1952) showed that mono- and di-iodotyrosine were formed when iodine was supplied to *Laminaria flexicaulis*, whilst Coulsen (1955), on the basis of paper chromatographic evidence, suggested that 3:5-diiodotyrosine, 3:5-diiodothyronine, thyroxine and thyronine all may be components of the protein of the red alga, *Rhodomenia palmata*. Scott (1954) utilized radioactive iodine (I^{131}) in his experiments and so made the detection of iodoamino acids more sensitive. He showed that *R. palmata*, *Ulva lactuca* and *L. digitata* incorporated radioactive I^{131} into compounds which were chromatographically identical with mono- and di-iodotyrosine and that the first two seaweeds also formed traces of an active substance corresponding with triiodothyronine. I^{131} did not appear to be incorporated into organic compounds by *Fucus vesiculosus* or in the stipe of *L. digitata*.

The aim of the present study was to determine whether angiosperms as well as marine algae possess the ability to incorporate iodine into organic compounds, and in particular into iodoamino acids. The plants used were of two types:

salt-marsh plants were represented by *Salicornia* and *Aster*, whilst barley and mung bean were used as examples of mesophytes. Similar previous work with mesophytes has been reported briefly by Tong and Chaikoff (1955). They showed that radioactive I^{131} was assimilated into mono- and di-iodo-tyrosine by the fresh water alga, *Nitella*, but they were unable to demonstrate any incorporation of iodine into organic compounds when *Lemna* fronds or horse-radish root slices were supplied with labelled iodine. Their experiments were of only four hours duration and normally used non-growing, excised tissues. In contrast the present experiments were performed with young, intact growing plants, *i.e.* using conditions that might be expected to be optimum for the successful demonstration of the possibility of iodine incorporation into organic compounds.

Methods

The overall procedure was to supply radioactive I^{131} as iodide to growing plants for various periods of time, when each batch of plants was divided. Approximately half of each set of plants were used for an examination of the aqueous-ethanol-soluble iodine-containing compounds, whilst protein samples were isolated from the remaining plants. These proteins were subjected to alkaline hydrolysis to release any iodoamino acids present. I^{131} -containing compounds in the different fractions were detected by the radioautographic technique.

Supply of I^{131} to salt-marsh plants. Young plants of *Salicornia perennis* and *Aster tripolium* (fresh weight about 0.2 g per plant) were obtained from a salt-marsh; mud was washed carefully from their roots using sea-water. 10 mc of a solution of I^{131} (supplied by the Radiochemical Centre, Amersham, as iodide in 1.0 ml of weak sodium thiosulphate solution, pH 8–10, total solids not exceeding 1 mg/ml) was added to 80 ml artificial sea-water (Pringsheim, 1946). Twelve plants of each type were supported on muslin over separate small containers and 40 ml of the I^{131} -containing solution was added to each. Uptake of I^{131} by the roots was allowed to continue for 3 days at 20°C under natural summer light conditions.

Supply of I^{131} to mesophytes. Batches of 12 seeds of barley (*Hordeum vulgare* var. Proctor) and mung bean (*Phaseolus aureus*) were each placed in 0.6 ml of a solution containing I^{131} (above solution diluted threefold) and germination was allowed to proceed at 23–25°. After 24 hours the seeds were rinsed and planted in expanded mica (vermiculite). Growth was allowed to continue for a further 5 days at the same temperature.

Separation of soluble iodine-containing compounds. Aqueous-ethanol-soluble compounds were obtained by macerating the plants in 75 % (v/v) ethanol (10 ml per g fresh wt. plant material). Extraction continued for 24 hours at laboratory temperature and was repeated twice. The extracts were combined and evaporated to dryness in vacuo. After redissolving the residues in distilled water, aliquots of the solutions were applied to chromatograms either (a) directly, or (b) after treatment on a cation-exchange resin column (Fowden and Webb 1958). Treatment (b) was used to remove most of the I^{131} -iodide, and leave a solution in which I^{131} was present

mainly in cationic substances, including iodoamino acids. The volumes of the final extracts applied to the chromatograms were adjusted to be equivalent to 0.1 g fresh wt. plant material.

Separation and hydrolysis of protein fractions. Protein fractions were prepared by macerating plants in an alkaline borate solution (about pH 9.0) and after removal of cell debris, the proteins were precipitated by adjusting to pH 4.5. Fuller details of the maceration technique and the subsequent treatment of the crude protein precipitates have been given for daffodil leaves (Bryant and Fowden 1959). The protein samples were hydrolysed using 5 N Ba(OH)₂ solutions at 100° for 20 hours. Barium was removed as sulphate and carbonate and the residual solutions of amino acids were concentrated. Aliquots equivalent to 2 mg of original protein preparation were applied to paper chromatograms.

Chromatography and radioautography. Paper chromatograms were prepared on Whatman no. 3MM chromatographic-grade filter-paper sheets. They were run first in 75 % phenol-aqueous NH₃ and the *n*-butanol-acetic acid-water mixture of Partridge (1948) was used as the second developing solvent. Development times were adjusted such that the final size of the chromatograms was 25×20 cm. Radioautographs were prepared by placing dried chromatograms in contact with Kodirex X-ray film for 3 weeks. The chromatograms were sprayed finally with ninhydrin reagent to locate the amino acids. Unlabelled iodoamino acids (3:5-diiodotyrosine, 3:5-diiodothyronine, 3:5-triiodothyronine and thyroxine) were added to certain chromatograms to act as marker substances.

Assays of radioactivity. These were made at various stages to determine the amounts of I¹³¹ absorbed by the plants. The activity present as iodide and in the soluble iodine-containing compounds and protein fractions of the plants were also determined. A gas-flow (methane) proportional counter attached to a scaler was used for counting. Corrections were applied to allow for the natural decay associated with the relatively short half-life of I¹³¹.

Results and Discussion

The *Salicornia* plants (2.0 g fresh wt.) absorbed 8.8 % of the I¹³¹ supplied in 3 days. The *Aster* plants (2.3 g fresh wt.) were less efficient in concentrating iodide and only absorbed 1.7 %. The bulk (98—99 %) of the iodide-I¹³¹ absorbed remained in this form in both plants at the end of the 3 day period. The absolute activity present in protein was very similar for both plants, but when expressed in terms of the total I¹³¹ absorbed, *Aster* protein contained 1.36 % and *Salicornia* protein only 0.29 % of this activity. The amounts of I¹³¹ associated with soluble, non-protein compounds were considerably smaller than those bound to protein.

The method by which I¹³¹ was supplied to the mung bean and barley plants naturally resulted in a more efficient absorption of the radioactivity. Approximately 90 % of the I¹³¹ supplied to the bean seeds and 48 % of that supplied to the barley was imbibed. Assimilation of I¹³¹ into the plant proteins was considerably less in these plants than for the salt-marsh plants

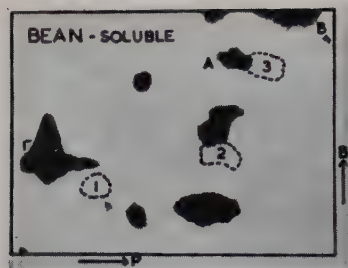


Figure 1. Radioautograph prepared from a chromatogram of the cationic fraction of an aqueous-ethanol-soluble extract of mung bean seedlings supplied radioactive I^{131} -iodide. $\rightarrow P$ indicates direction of phenol- NH_3 solvent development and $\rightarrow B$ that of the *n*-butanol-acetic acid-water solvent flow. The designated radioactive spots are I^- , iodide; A, 3:5-diiodotyrosine; B, 3:5-diiodothyronine. Other undesignated spots represent unidentified radioactive I^{131} -containing substances. The dotted areas are the positions occupied by some common amino acids for reference purposes: 1, glutamic acid; 2, alanine; 3 valine.

and even higher percentages of I^{131} remained as iodide at the end of the growth period. Only 0.058 % and 0.0054 % of the activity absorbed by the mung bean and barley seeds respectively was found to be associated with protein. Negligible activity was present in the aqueous-ethanol-soluble compounds of barley seedlings, but in contrast the soluble compounds of the mung bean seedlings contained approximately 0.2 % of the total I^{131} absorbed, *i.e.* more than three times the activity present in the protein of the same plants.

Figure 1 illustrates the radioautograph obtained from the soluble compounds of the mung bean plants. The sample applied to the chromatogram had been treated on a cation-exchange resin column, but it proved very difficult to wash away the last traces of I^{131} -iodide radioactivity. This radioautograph indicated the presence of a considerable number of iodine-containing compounds (cationic in character) in mung bean seedlings. Only two of these substances (spots A and B) have been identified; A was inseparable from added 3:5-diiodotyrosine and B ran coincidentally with 3:5-diiodothyronine. The most strongly radioactive substance appeared below alanine (spot 2); in position it was almost coincident with that occupied by glutamine. At present its chemical nature remains unknown as do those of the other undesignated spots on the radioautograph (Figure 1). The corresponding radioautographs of the aqueous-ethanol-soluble compounds of *Salicornia* and *Aster* showed the presence of spots A and B, but their intensities were considerably weaker than in mung bean. A further radioactive spot C (tentatively identified as 3:5:3'-triiodothyronine; see later protein radioautographs) was present in the extracts of both *Salicornia* and *Aster*, but no significant quantities of any other I^{131} containing compounds could be detected. No radioactive compounds other than iodide were found in soluble extracts of the barley seedlings. In all cases the quantities of radioactive I^{131} -containing substances present on the chromatograms were insufficient to give positive colour responses after ninhydrin treatment, even for spots A, B and C which were known to be amino acids.

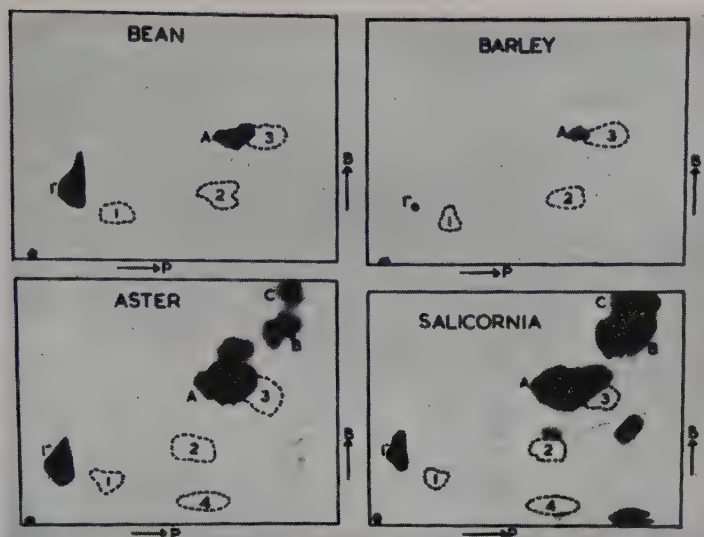


Figure 2. Radioautographs prepared from protein hydrolysates of plants supplied radioactive I^{131} -iodide. $\rightarrow P$ indicates direction of phenol- NH_3 solvent development and $\rightarrow B$ that of the *n*-butanol-acetic acid-water solvent flow. The designated radioactive spots are: I⁻, iodide; A, 3:5-diiodotyrosine; B, 3:5-diiodothyronine; C, 3:5:3'-triiodothyronine. Other undesignated spots represent unidentified radioactive I^{131} -containing substances. The dotted areas are the positions occupied by some common amino acids for reference purposes; 1, glutamic acid; 2, alanine; 3, valine; 4, glutamine.

The radioautographs obtained from the chromatograms of the hydrolysed proteins of each plant are shown in Figure 2. It is obvious that the proteins of the salt-marsh plants contained a far greater variety of iodoamino acids than did those of the two mesophytes. 3:5-diiodotyrosine (spot A) was the only iodo-compound associated with the proteins of mung bean and barley seedlings (the only other active spot on these radioautographs was due to iodide contamination). The diiodotyrosine arising from the mung bean protein contained approximately five times the activity of that released from the barley protein. Diiodotyrosine also formed the most active component arising from both the *Salicornia* and *Aster* protein fractions. In addition these proteins both contained the active substances B and C which have been identified provisionally as 3:5-diiodothyronine and 3:5:3'-triiodothyronine respectively. The activities of B and C relative to that of diiodotyrosine (A) were markedly greater for *Salicornia* protein than for the protein of *Aster*. A number of other radioactive spots are visible on both of these radioautographs indicating that in the salt-marsh plants there are other iodo-amino acids associated with protein that so far remain unidentified. The

amounts of diiodotyrosine present in the proteins were sufficient to react weakly with the ninhydrin reagent.

The identifications of B and C as di- and tri-iodothyronine respectively were based on their cochromatography with authentic samples of the latter substances. No iodoamino acid other than diiodothyronine moved to the same position as B in the normal pair of solvents used here, but both triiodothyronine and thyroxine moved to a position coincident with C. The identity of C with triiodothyronine was confirmed using the *n*-butanol-dioxan-2 *N* ammonia solvent of Gross, Leblond, Franklin and Quastel (1950), which effects a separation of this iodoamino acid from thyroxine.

There are a number of reports describing the partial instability of certain iodoamino acids during alkaline hydrolysis and the possibility that iodoamino acids are formed from free iodide and tyrosine under these conditions. A fairly detailed study of anomalous compounds that may arise in this way has been made by Scott (1954). He found by using radioactive I^{131} that free amino acids (*e.g.* tyrosine, diiodotyrosine, etc.) could be iodinated under these alkaline conditions to give a number of I^{131} -labelled substances. If such iodinations occurred between any I^{131} -iodide absorbed onto the protein fractions used in the present experiments and tyrosine formed during the hydrolysis of the proteins, then great caution would have to be exercised before any iodoamino acids resulting could be regarded as true protein constituents and not just artefacts. Fortunately Scott observed that if radioactive I^{131} was added during the alkaline hydrolysis of certain algal proteins, no formation of radioactive iodoamino acids occurred.

This type of check was performed for each of the plant proteins used in the present experiments. Samples of proteins were isolated from plants that had not been supplied with radioactive I^{131} during their growth. These control protein samples were then hydrolysed after I^{131} had been added to give an activity equivalent to that found in the active experimental protein samples. When radioautographs were prepared from the hydrolysates of these control protein samples, radioactivity was present in only one spot corresponding to the added I^{131} -iodide. The possibility that the iodoamino acids associated with the plant proteins arose as artefacts would seem then to be discounted. The fact that the relative amounts of different iodoamino acids varied markedly for the different plant proteins, all of which received the same hydrolytic treatment, was in itself another factor indicating the impossibility of them all arising by artefact iodination reactions.

The ease with which triiodothyronine and thyroxine can be coprecipitated with protein (Wilkinson, Sprott, Bowden and Maclagan 1954, Maclagan, Bowden and Wilkinson 1957) makes it somewhat uncertain whether iodoamino acids shown to be associated with the protein fractions should be

regarded as integral components of the protein molecules or merely as molecules absorbed upon the surfaces of the proteins during their precipitation. Other iodoamino acids do not appear to be so readily coprecipitated together with protein and, in the case of the iodo-compounds present in the aqueous-ethanol-soluble extract of mung bean seedlings, general coprecipitation obviously did not occur for the protein fractions contained only diiodotyrosine. At least some of the iodoamino acids shown to be associated with protein are then probably present entirely as components of the protein; other acids may be associated with protein both as a direct constituent and as surface-absorbed molecules. Since the relative quantities of the iodoamino acids present in protein are small compared with the amounts of other amino acids, one may infer that the iodoamino acids probably occur in only a few types of protein molecules, which themselves may play a definite role or have a special localization within the plant cells.

The difficulty of deciding whether the iodoamino acids are true protein components should not be allowed to obscure the more important finding, namely that these angiosperms have the ability to introduce iodine into amino acids in the same manner as that previously known for marine algae.

Summary

Radioactive I^{131} -iodide has been employed to determine whether certain angiosperms have a similar ability to assimilate iodine into organic compounds (iodoamino acids) as that shown by some large red and brown algae. Two types of plants were used: salt-marsh plants were represented by *Salicornia perennis* and *Aster tripolium*, whilst barley and mung bean seedlings were used as examples of mesophytes. Whilst the great majority of the total I^{131} absorbed still remained as iodide several days after the beginning of its supply, small amounts had become associated with the proteins of all plants. The small quantities of I^{131} found in the barley and mung bean proteins were present entirely in the form of 3:5-diiodotyrosine. In contrast hydrolysis of the *Salicornia* and *Aster* proteins produced several I^{131} -labelled iodoamino acids, including 3:5-diiodothyronine and 3:5:3'-triiodothyronine in addition to diiodotyrosine, together with small amounts of several other uncharacterized active substances.

The presence of I^{131} in aqueous-ethanol-soluble substances of the plants was also examined. In general little activity was found in these compounds, but mung bean extracts proved an exception. Here the quantity of I^{131} in the soluble compounds considerably exceeded that in the protein of the plant.

and radioautography showed the presence of the three iodoamino acids listed above, together with several other as yet unidentified compounds.

It would seem that the ability of the salt-marsh plants to incorporate iodine into organic compounds parallels, at least in the variety of compounds formed, that of the marine algae: Mesophytes possess a more restricted capacity to synthesize iodoamino acids.

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Some Further Data on Pyridoxamine-deficient Mutants in *Ophiostoma*

By

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In previous communications (Wikberg 1959 a, 1959 b) an account has been given of some physiological and genetical characteristics of two pyridoxamine-deficient mutant strains, W 233-2 and W 366-10, of *Ophiostoma*. Recently one more pyridoxamineless mutant, No. 2855, has been isolated by Professor N. Fries. In contrast to the ultraviolet induced W 233-2 and W 366-10, No. 2855 has been obtained through X-ray induction. In view of the rarity of this mutant type is seemed justified to perform some experiments also with No. 2855. In this paper a report of these experiments is given and a comparison is made with the strains previously described. If not specially mentioned in the text the methods used in growth and mating experiments were the same as those used in the previous work.

Results and Conclusions

Genetical data. In a cross of No. 2855(+) to wild type 1(—), the characters 'pyridoxamineless' and 'wild' segregated in the expected ratio 1:1. Out of 23 isolated ascospores 11 were mutants, that is, they responded to pyridoxamine but not to pyridoxine, and 12 were wild type, growing with both pyridoxine and pyridoxamine.

A low percentage of wild-type mycelia occurred in the progeny of a cross between W 233-2 and W 366-10 (Wikberg 1959 b). They were believed to be recombinants, and thus W 233-2 and W 366-10 are non-allelic.

In a corresponding manner No. 2855(+) has been crossed to W 233-2-3(—), pyridoxamine-less, and to W 336-10-14(—), pyridoxamine-less. W 233-2-3(—) and W 366-10-14(—) are f_1 substrains of W 233-2(+) and W 366-10(+), respectively. In a plating out of an ascospore suspension from both crosses on minimal agar medium containing pyridoxine, mycelia appeared, which also in further tests behaved as prototrophs. From the cross $2855(+) \times W 366-10-14(—)$ 146 ascospores were isolated. None of the mycelia from these responded to pyridoxine at 30°C. When 32 of them were transferred to 20°C, started growing thus being of the same type as the temperature-sensitive W 366-10-14, while 16 of the strains did not respond to pyridoxine at this temperature; that is, they behaved as No. 2855 (see below).

With a large number of ascospores from the same cross ($2855 \times W 366-10-14$) a screening for prototrophic strains has been made. Some method for the large scale isolation of 1-ascospore mycelia has not yet been worked out for *Ophiostoma*. In this fungus the ascospores are liberated from the perithecia in clumped aggregates and do not separate readily from each other when suspended in water. The observation was made, however, that the spore lumps could be disintegrated by catching the exuded drops from the necks of the perithecia in a loop with potassium chloride solution (about 1 M). Through this procedure the majority of the spores were separated from each other. They were then immediately transferred into sterile water.

The screening experiment was performed in the following way. The ascospores were plated out on minimal pyridoxine medium, 5 plates in each series. A count of the total number of ascospores was obtained from platings (5 plates) in minimal pyridoxamine medium. From a total of $1.75 \cdot 10^4$ spores 136 mycelia or 0.8 per cent were wild types. Since an unknown number of the counted mycelia on the pyridoxamine medium have developed from more than one ascospore this figure is probably too high. Sixteen of the prototrophs were transferred on pyridoxine minimal and still behaved as wild types. However, they have not been crossed to standard wild type as yet. Conclusive proof of recombination would, of course, be the finding of the expected pyridoxamine-less double mutants. Since the growth characteristics of these are unknown, it has not been possible to search for them. Since monoconidial mycelia had been used as inoculating material in the crosses, it is probable that spontaneous back-mutations did not interfere in these results (see below). On basis of the results above it is assumed that the mated strains W 366-10-14 and No. 2855 are non-allelic. However, since direct proof of recombination is lacking the possibility must still be considered that some mechanism other than recombination and back-mutation may be responsible for the occurrence of the wild-type mycelia.

Table 1. *Effect of compounds of the vitamin B₆ group on the growth (extinction values Z) of No. 2855, pyridoxamine-less, in shaken tube cultures. $Z = (e_v - e_0) \times 10^3$, where e_0 = extinction value of the inoculated tube at time 0. e_v = extinction value of the tube at time v.*

Vitamin added	mμmol per 10 ml	Z-values after incubation in days					
		3	4	5	6	7	8
Pyridoxamine	0.03	—	130	—	160	—	—
	0.1	—	410	—	540	—	—
	0.3	—	910	—	910	—	—
Pyridoxal	1	—	0	0	0	—	—
	3	—	60	130	180	—	—
	10	—	150	310	330	—	—
	30	—	80	590	930	—	—
	100	—	80	820	1,340	—	—
Pyridoxamine + Pyridoxine	0.3 + 1	—	810	820	850	—	—
	0.3 + 10	—	660	740	740	—	—
	0.3 + 30	—	0	0	0	—	—
Pyridoxamine phosphate	0.1	0	0	40	40	70	115
	0.3	0	53	220	450	740	850
	1	260	720	1,180	—	—	—
Pyridoxal phosphate	0.3	0	0	0	+	+	+
	1	+	100	310	700	980	—
	3	210	760	1,260	—	—	—

An estimation of the occurrence of spontaneous back-mutations in W 233-2, W 366-10, and No. 2855 has been made by using the procedure described by Zetterberg and Fries (1958). A series of 10 plates with $2 \cdot 10^7$ conidia per plate in minimal agar medium supplied with pyridoxine (100 mμmol per plate) were incubated at 30° for 5 days. By this method it was found that, for W 233-2 and W 366-10, prototrophs occurred in a low frequency with 2 and 5 mycelia respectively in one experiment for each strain. For No. 2855 no prototrophs were found in three different experiments.

Growth experiments The effect of all the compounds of the vitamin B₆ group on the growth of No. 2855 has been studied in shaken tube cultures at 30°. The results have been summarized in Table 1, where growth is indicated as extinction values Z. Pyridoxine alone did not promote growth at any concentration tested. The effect of pyridoxamine is also similar to the effect in W 233-2 and W 366-10 (Wikberg 1959 a). But as judged from the final extinction values, No. 2855 does not utilize the vitamin with the same efficiency as do the other two mutants. For pyridoxal a threshold concentration of about 3 mμmol per 10 ml is required for growth to occur. Therefore, No. 2855 does not differ from the other two mutants as concerns the reaction towards pyridoxal.

If the extinction values obtained with pyridoxamine phosphate (Table 1)

Table 2. *The effect of pyridoxal at 20 and 30°C and pyridoxamine at 20° on the growth of pyridoxamine-deficient No. 2855. Dry weights of mycelium in mg after 6 days. The values are the mean of 2—4 flasks.*

Vitamin added	μmol per 20 ml	20°	30°
Pyridoxal	0.3	1.2	1.2
	1	1.0	0.6
	10	3.0	2.1
	100	17.4	2.3
Pyridoxamine	0.3	25.1	—
	1	35.1	—
	10	65.8	—

are compared with those of the pyridoxamine series it is seen that on a molar basis the activity of pyridoxamine phosphate is lower and that it is utilized at a lower rate than is pyridoxamine. Similar results were found for No. 51, wild type, as well as for W 233-2 and W 366-10 (Wikberg 1959 a). With respect to the reaction towards pyridoxamine phosphate, No. 2855 seems to represent an intermediate type between No. 51 and the other pyridoxamine-deficient strains. The 'minimal active dose' for No. 51 is about 0.01—0.03 μmol per 10 ml, for No. 2855 0.1 μmol and for W 233-2 and W 366-10 about 1 μmol.

The reaction of No. 2855 towards pyridoxal phosphate is similar to that towards pyridoxamine phosphate. The threshold concentrations required were for No. 51 0.01—0.03 μmol, for No. 2855 0.3—1.0 and for W 233-2 and W 366-10 about 10 μmol per 10 ml.

An antagonistic interaction between pyridoxine and pyridoxamine was found in W 233-2 and W 366-10. The same phenomenon was noted in No. 2855 (Table 1). The pyridoxine-pyridoxamine ratio for total inhibition of growth is also the same as for the other pyridoxamine-less mutants, *viz.* about 100 : 1.

The activity of pyridoxine, pyridoxal and pyridoxamine was also studied at 20° in 100 ml flasks containing 20 ml medium. Pyridoxine is not active. No. 2855 is not temperature-sensitive in contrast to W 366-10 with respect to pyridoxine. From the dry weights of the pyridoxal series (Table 2) it is seen that for the highest concentration used, *viz.* 100 μmol per flask, more mycelium is produced at 20 than at 30°. The interpretation can be made that the partial metabolic block which prevents the utilization of pyridoxal is to some degree relieved under these conditions.

Summary

An X-ray induced pyridoxamine-deficient mutant in *Ophiostoma* has been studied, and comparisons are made with two previously described pyridoxamine-less strains as regards its genetical and physiological characteristics.

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The Localisation and Properties of Pectin Methylesterase of *Avena* Coleoptiles

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I. Introduction

The pectic substances possibly play an important part in the control of cell wall properties in tissues where rapid changes in structural rigidity are occurring. In certain tissues there is a correlation between changes in the pectins and the effects of auxins. Wilson and Skoog (1) measured the uronide composition of tobacco pith sections stimulated into rapid cell enlargement with indole-3-acetic acid (IAA). Growth by cell enlargement in this tissue appears to be correlated with the synthesis and utilisation of uronides soluble in 70 % ethanol. Bryan and Newcombe (2), using the same tissue, observed that IAA-induced cell enlargement was accompanied by an increase in pectin methylesterase (PME) activity to almost twice the value (on a nitrogen basis) for control sections after 120 hours.

Kögl (3) analysed the cell wall composition of roots and shoots of *Avena* grown in liquid medium. Addition of IAA to the medium inhibited the growth of the roots but not the shoots. No differences were observed in the wall composition of shoots but in the presence of IAA, the total pectin content of roots was considerably reduced, the main effect being on the water-soluble uronides. Increased calcium binding in IAA-treated roots was also observed. This may have been due to decreased methyl esterification of the pectic carboxyl groups, as Keller and Deuel (4) have shown that 70 to 80 % of the cation binding capacity of killed roots could be attributed to the pectic carboxyl groups.

Sacher and Glasziou (5) observed changes occurring in bean endocarp segments maintained on wetted filter paper. Liquid-logging of intercellular air spaces was evident in 2 $\frac{1}{2}$ —3 days and an exudate appeared at the upper tissue surfaces. Cellular dissociation was usually complete by the fifth day. Analysis of the exudate showed the presence of PME and water soluble uronides. All these changes were essentially prevented by addition of an auxin (IAA or α -naphthalene acetic acid). Observations by Sacher (6) on a number of plant species have shown that the sequence of events discernable microscopically in abscission zones is similar to those proceeding in bean endocarp.

Osborne (7) determined the amount of PME in the supernate from tissues of *Phaseolus vulgaris* ground in distilled water. A sharp gradient of enzyme activity was observed across abscission zones located in the proximal and distal pulvini. The results suggested that the advent of abscission may be linked with the steepness of the gradient, abscission occurring when the gradient disappeared. Treatments which accelerated abscission also accelerated the disappearance of the gradient. Conversely, treatments with auxins which retarded abscission also maintained the gradient of PME activity across the abscission zone.

In other tissues gross changes in the pectic substances may occur without any known correlation with auxin activity. Dame, Luh and Marsh (8) showed that during the ripening of pears, increasing softness of the fruit is correlated with a conversion of protopectin to water-soluble pectin, the solubilization of the pectic substances being accompanied by the appearance of methanol in this tissue.

These results demonstrate that metabolic processes, almost certainly enzymatic which involve pectic substances occur in cell walls. The problem arises whether a group of enzymes is located in the wall, and whether enzymes concerned with both catabolic and anabolic processes are included in the group. There has been considerable divergence of opinion on the degree of intimacy of contact between wall and cytoplasm. The electron micrographs of Buvat and Lance (9) and Setterfield, Stern and Johnston (10) on higher plants clearly show the presence of a similar type of double membrane at the cytoplasm-wall interface as has been observed surrounding mitochondria, chloroplasts, and nuclei. It appears likely that the presence of such a barrier would confer a degree of autonomy to the wall requiring the presence of wall enzymes to catalyse structural changes and perhaps certain synthetic processes.

The difficulty of obtaining conclusive results on the localization of an enzyme is well recognised. Convincing evidence for the presence of invertase activity at the surfaces of intact cells of *Canna* leaves has been presented by Hassid (11), and by Burström (12) for the localization of invertase activity

in the free space of wheat roots and coleoptiles. Evidence presented by Gall (13) suggested the possibility of an extracellular starch hydrolysing enzyme in cultures of bean stem segments, the quantity (or activity) of which is increased by 2,4-dichlorophenoxyacetic acid in the culture medium. The role of these enzymes is not clear; invertase may be concerned with sugar translocation and uptake into the cell.

Recent studies by Kivilaan, Beeman and Bandurski (14) indicate that uridine diphosphoglucose pyrophosphorylase, adenosine triphosphatase, and pyrophosphatase activities are associated with corn coleoptile cell wall fragments isolated in non-aqueous medium (glycerol).

Previous work of Glasziou and Inglis (15) on the PME of tobacco pith sections showed that this enzyme could readily be extracted from intact sections by dilute salt solutions. The enzyme was partitioned between the sections and the solution and came to an equilibrium which could be altered by the presence of an auxin. The time taken to attain equilibrium was between 10 and 15 minutes for 1 to 2 mm sections suggesting that no serious diffusion barriers were interposed between the enzyme in situ and the extracting solution. As the extraction of the enzyme could be very strongly influenced by the type and concentration of cation and by the pH of the external solution, it is probable that this enzyme is located in the free space.

This paper presents evidence for the localization of the PME of *Avena* coleoptile sections in the free space, and describes some of the properties of the enzyme.

II. Materials and Methods

Avena seedlings were grown in the manner of McRae and Bonner (17). Coleoptiles were harvested after 85–90 hours, the apical 2–3 mm removed, and the next 5 or 10 mm used as experimental material. For all experiments except those indicated in the text the primary leaves were removed.

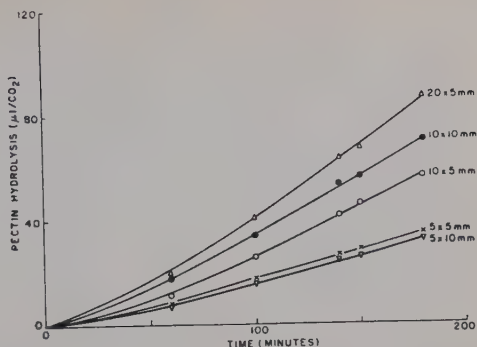
Extraction of PME. In a few instances, extraction and assay of PME was carried out in the one operation, but usually this was not possible and extraction was carried out in salt solution with a sodium bicarbonate- CO_2 buffer system at constant temperature in a Dubnoff shaker. The gas phase was 95 % N_2 , 5 % CO_2 .

Assay of PME. The method described previously (15) which utilises standard Warburg manometric procedures for the measurement of acid production was used throughout. For all assays the salt concentration was adjusted to give optimum enzyme activity.

PME units. For the purpose of this work, the PME unit was defined as that amount of enzyme which gave a rate of pectin hydrolysis measured as 1.0 μl CO_2 evolved per hour from a bicarbonate- CO_2 buffer at pH 7.0 and 30°C. This unit is equivalent to a rate of 4.5×10^{-2} microequivalents of pectin ester bonds hydrolysed per hour.

Pectin. The pectin used for PME assays was kindly supplied by Dr. Eugene Jansen. The specifications were grade 180, 70–75 % esterified slow-set citrus pectin, ash content 0.8 %.

Figure 1. *The hydrolysis of pectin by intact Avena sections.* Sections (primary leaves not removed) were placed in 0.1 M NaCl and 0.5 % pectin; bicarbonate-CO₂ buffer, pH 7.0; Final vol. 3.0 ml; 30°C.



III. Results

1. *The effect of length and number of sections on the rate of extraction of PME.* The hydrolysis of pectin against time is plotted in Figure 1 for different numbers and lengths of section per treatment. These results made it clear that the PME activity measured was more closely related to the number of sections present than to the total amount of material used, suggesting that the enzyme came either from damaged cells at the cut ends or else diffused out of the tissue via cut ends and not across the cuticle. As with tobacco pith (15) on removal of the *Avena* sections, all of the PME activity remained in the extracting solution. In Figure 2, a comparison is shown of the time course of extraction of PME from 1.0 and 2.5 mm coleoptile sections. In this experiment the enzyme was extracted in a buffered salt solution (without pectin), and the PME activity subsequently determined. If the enzyme came from damaged cells alone, it would be expected that sections of

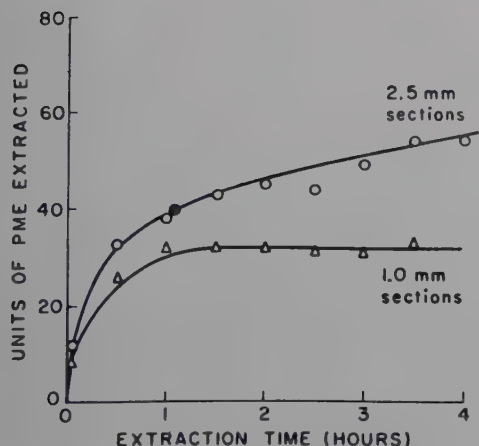


Figure 2. *The time course of extraction of PME from 1.0 and 2.5 mm coleoptile sections.* 50 sections per treatment were extracted in 0.1 M NaCl; bicarbonate-CO₂ buffer, pH 7.0; final vol. 3.0 ml; 26°C. The PME activity of the extracts was assayed manometrically. ● = Theoretical value at $t_{1/2}$ for 2.5 mm sections.

Table 1. *The extraction of pectin methylesterase from 1.0 mm Avena coleoptile segments.* 50 sections 1.0 mm in length were extracted for 1 hour in 0.1 M NaCl at pH 7.0, 26°C., N₂, 5 % CO₂. The extract was removed and the extraction repeated. At the conclusion of the second extraction, the sections were homogenised in 0.1 M NaCl. The PME activity of each fraction was assayed manometrically. Results are means of duplicate experiments.

Fraction	Units of PME activity	PME content as percentage of total
First extract	53	45
Second extract	22	18
Tissue homogenate . . .	44	37

different lengths would give the same result. Obviously this is not so, and the enzyme must diffuse from deeper in the tissue. The time taken to attain diffusion equilibrium for 1.0 mm coleoptile segments was between 1.0 and 1.5 hours. If diffusion through the cut ends only is involved, Philip (18) has shown that the coleoptile may be treated as a sheet and the half-time for the attainment of diffusion equilibrium is a function of the square of the section length. For 1.0 mm sections $t_{1/2}$ is about 10.5 minutes, so that for 2.5 mm sections $t_{1/2}$ should be about 66 minutes, and about 40 units of PME should have diffused from the tissue. This point is plotted in Figure 2 and shows reasonable agreement with the experimental data.

2. *Diffusion and adsorption characteristics of Avena PME.* The results for 1.0 mm sections (Figure 2) enable an estimate to be made of the diffusion constant for PME in the Avena sections. The appropriate equation from (18) is

$$D = 0.195 a^2 / t_{1/2}$$

where D is the diffusion constant and a is half the length of the section in cm. For PME diffusing through a coleoptile, D is approximately $8 \times 10^{-7} \text{ cm}^2 \text{ sec}^{-1}$. The dimensions and molecular weight of this enzyme are not known. However, for a wide range of molecular sizes and shapes the diffusion constant for proteins in water is from 10^{-6} to $10^{-7} \text{ cm}^2 \text{ sec}^{-1}$. It can therefore be concluded that in moving through the tissue, PME moves as a protein in water and does not pass through any serious barrier to diffusion.

A further check was made to show that the bulk of the PME was not derived from damaged cells. 1.0 mm coleoptile sections were extracted in 0.1 M NaCl for 1 hour, the solution containing the enzyme removed and the sections extracted a second time in the same way. The tissue was then homogenised and the PME content of each fraction determined. The results in Table 1 show that 63 % of the PME content was removed in 2 extractions. Cytological examination showed that for 1.0 mm sections about 20 % of the cells were cut.

Table 2. *The extraction of PME from coleoptiles in non-aqueous medium.* 50×10 mm sections (primary leaves removed) were frozen and ground in powdered dry ice. 6.0 ml glycerol was added, mixed and filtered. In experiment 2 the residues were extracted a second time with glycerol. PME assayed manometrically.

Experiment	Fraction	Units of PME activity	PME content as percentage of total
1	glycerol filtrate.....	80	5.9
	cell residues	1,280	94.1
2	first glycerol filtrate	56	4.9
	second glycerol filtrate	—	—
	cell residues	1,092	95.1

The results of Table 1 allow a calculation to be made of the proportion of adsorbed to soluble enzyme in the free space of *Avena* coleoptiles *in vivo*, the assumptions being that the pH and cation concentration in this zone is approximately equivalent to that of the extracting medium. Previous results on tobacco pith (15) and the results presented herein, indicate that diffusion of PME from the tissue may be treated as

Adsorbed enzyme \rightleftharpoons free enzyme in tissue \rightleftharpoons enzyme in external solution.

When diffusion equilibrium is reached the concentration of enzyme in solution in the tissue must be the same as that in the external solution. The volume of the free space may be calculated assuming that it occupies about 25 % of the total tissue volume (16) and from these data the ratio of adsorbed to free enzyme can be obtained. It is estimated that 0.5 % of the PME content of the coleoptile is in solution in the wall zone.

3. *Isolation of PME from coleoptiles in non-aqueous medium.* To avoid the necessity of making assumptions on the pH and ionic conditions in the wall zone and with the hope of obtaining more correct results on the ratio of adsorbed to free enzyme *in vivo*, an estimate of the free PME was obtained in the following manner. Sections were rapidly frozen in powdered dry ice, then ground in dry ice to a fine powder. Glycerol was added, the tissue ground a second time, and then filtered through Whatman 50 filter paper. A measured volume of the filtrate was dialysed to remove glycerol, and the PME activity of the dialysate and the residues determined. In a repeat experiment, the residues were extracted a second time with glycerol. The results (Table 2) show that 5—6 % of the total PME may be removed on the first extraction with glycerol and that no more enzyme is obtained in a subsequent extraction.

The assumption inherent in this method is that the rapid dilution of cell water by glycerol during thawing and grinding will prevent movement of

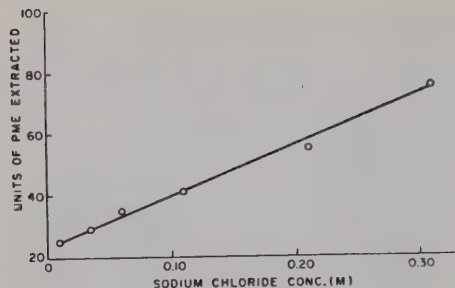


Figure 3. The effect of salt concentration on the rate of extraction of PME. 20×5 mm sections (primary leaves not removed) were extracted in salt solutions as shown: bicarbonate- CO_2 buffer, pH 7.0; final vol. 3.0 ml; 80 minutes at 26°C . The PME activity was assayed after adjustment of the NaCl concentration to give optimum activity.

enzymes from cell bodies or surfaces with which they are normally associated. Enzymes soluble *in vivo* should be found in the glycerol filtrate. The quantity of free enzyme estimated in this way is ten times higher than that estimated from diffusion equilibria. However, it is reasonable to conclude that in excess of 90 % of the PME of intact *Avena* coleoptiles is in the bound state. It is of interest that the amount of PME found in the glycerol extract would be capable of hydrolysing all of the methyl ester groups of the pectin of *Avena* sections in about 6 hours, if it was operating at maximum activity (calculated from data of Jansen, Jang, Albersheim and Bonner, 19).

4. *The effect of different salts and salt concentrations on the extraction of PME.* Figure 3 shows the effect of increasing NaCl concentration on the rate of extraction of PME from 5 mm *Avena* shoots (primary leaves not removed in this experiment). In Table 3, the effectiveness of sodium, potassium and calcium chlorides on PME extraction from coleoptiles is compared, and in Figure 4 the effect of CaCl_2 on the extraction of PME and U.V. absorbing material from 1.0 mm coleoptile segments is given. The results show that the rate of extraction of PME is an approximately linear function of the salt

Figure 4. The effect of CaCl_2 on the extraction of PME and material absorbing in the ultra-violet from coleoptile sections. 20×5 mm sections were extracted in CaCl_2 as shown; bicarbonate- CO_2 buffer; pH 7.0 (0.02 M with respect to sodium ions); 60 minutes at 26°C . CaCl_2 levels adjusted to equal levels prior to PME assay and U.V. absorption determinations.

○—○ PME activity. △—△ optical density extract.

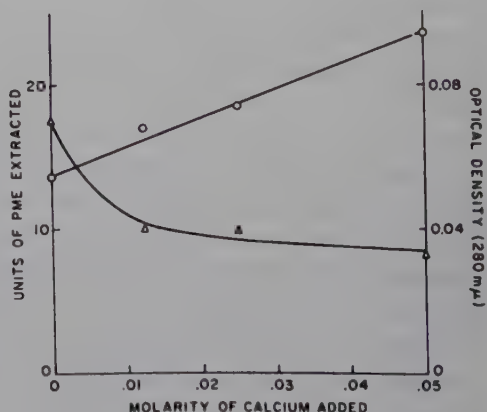


Table 3. *The effect of sodium, potassium and calcium chlorides on the rate of extraction of PME from coleoptiles.* 20×5 mm sections were extracted in 0.05 M salt solution at pH 7.0, N₂, 5 % CO₂. PME assayed manometrically. NaCl added to assay mixture to give 0.15 M cation concentration.

Cation	Concentration (M)	Ionic strength of salt	Units of PME extracted
Na	0.05	0.05	15
K	0.05	0.05	14
Ca	0.05	0.15	44

concentration, and that the three salts are equivalent in extraction efficiency when expressed on the basis of ionic strength, but that CaCl₂ is about three times better than NaCl or KCl on a molar basis. It should be emphasised that the results recorded are for the extraction of PME and not on the activity of the enzyme which is also affected by the type of cation present in the assay solution.

That many tissues become leaky when placed in distilled water or solutions containing monovalent ions is well known. Leakiness can often be prevented by the addition of small amounts of calcium. The fact that leakage of U.V. absorbing substances is reduced to a low, constant level by addition of small amounts of calcium while the amount of PME extracted increases linearly with increasing calcium concentration shows that leakiness and extraction of PME are not related phenomena.

5. *The effect of NaCl concentration on the activity of PME extracted from Avena coleoptiles.* The activity of PME from a variety of sources is altered by the salt concentration in the assay solution. According to Lineweaver and Ballou (19), the activity of PME at pH values below its isoelectric point is decreased due to the formation of salt linkages with the free carboxyl groups of the substrate. The salt activation is brought about by a competitive effect for the anionic groups of the substrate. In Figure 5 the effect of NaCl on the

Figure 5. *The effect of NaCl concentration on the activity of PME from coleoptile sections.* 2,500×1.0 mm sections were extracted for 1 hour in 40 ml 0.1 M NaCl. The extract was filtered and dialysed against two changes of 5 l distilled water for 24 hours. The PME activity at various salt concentrations was assayed on 1.5 ml aliquots. ○—○ Avena coleoptile PME. △—△ Alfalfa PME. Curve taken from the data of Lineweaver and Ballou (19).

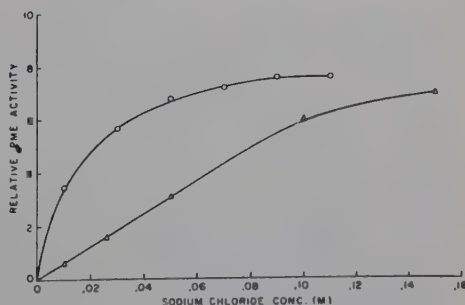


Table 4. *The distribution of PME between primary leaves and coleoptiles.* For homogenates, the tissue was ground in 0.1 M NaCl, allowed to stand 20 minutes, and an aliquot of the suspension used for enzyme assays. For PME extraction 20×1.0 mm sections were placed in 0.1 M NaCl buffered at pH 7.0 with bicarbonate-CO₂ for 100 minutes. PME in extract assayed manometrically.

System	Tissue	Units of PME	% of PME activity attributable to primary leaves
Homogenate	coleoptiles	50	} 45
	primary leaves	42	
Salt extract from intact sections at diffusion equilibrium	coleoptiles alone	12.5	} 46
	coleoptiles plus primary leaves	23	

activity of PME extracted from coleoptiles and alfalfa PME (data of Lineweaver *et al.*) is given. The *Avena* enzyme attains full activity at a considerably lower NaCl concentration than the alfalfa enzyme, half the maximum activity being obtained at about 0.01 M NaCl.

6. *The effect of O₂, N₂ and pectin on the extraction of PME.* The effect of O₂ or N₂ in the gas phase during the extraction of PME was examined to check the possibility that movement of the enzyme involved metabolic processes such as protein synthesis, membrane permeability, etc., likely to be affected by aerobic or anaerobic conditions. The results show no differences in the amount of PME extracted whether O₂ or N₂ was present in the gas phase.

The possibility that pectin in the tissue acted as a site of PME adsorption was investigated by placing pectin in the extracting solution. Any appreciable binding of PME to the pectin of the medium should increase the diffusion gradient and the amount of enzyme extracted. No effect of pectin was observed.

7. *The distribution of PME in primary leaves and coleoptiles.* In initial experiments the primary leaves were not removed from coleoptile sections. Experiments were therefore carried out to compare the diffusion of PME from sections with and without primary leaves. The results show (Table 4) that 45 % of the total PME of *Avena* sections is in the primary leaves and that there is a 46 % decrease in the amount of PME at diffusion equilibrium when the enzyme is extracted from 1.0 mm sections with the primary leaves removed. The extraction of PME from primary leaves is therefore similar to PME from coleoptiles.

IV. Discussion

The diffusion of PME from *Avena* coleoptile segments has the following characteristics. When salts are added to the bathing solution the enzyme diffuses from the interior of the tissue via the cut ends. The diffusion constant for the movement of PME out of the sections is that of a protein in water ($8 \times 10^{-7} \text{ cm}^2 \text{ sec}^{-1}$), indicating the absence of serious permeability barriers. Two thirds of the total PME from 1.0 mm coleoptile sections is extracted with two successive treatments in 0.1 *M* NaCl. The rate and amount of PME extracted is a linear function of the external salt concentration. Concentrations of salts as low as 0.01 *M* NaCl or 0.003 *M* CaCl₂ suffice to extract considerable quantities of PME. The addition of calcium ions to coleoptile segments placed in a solution buffered with potassium ions reduces the leakiness of the tissue (measured by absorption at 280 mμ) to a low level, and at the same time the amount of PME extracted increases in direct proportion to the calcium concentration.

These results show that most of the PME of *Avena* coleoptiles is localized in the free space which certainly includes the cell walls. Calculations from diffusion equilibria data and from enzyme isolation in non-aqueous media indicate that greater than 90 % of the PME is bound in the intact coleoptile. The question has been asked by Osborne (7) whether binding of PME is necessarily an inactivation. On kinetic grounds it would be expected that such would be the case where the substrate is a large, perhaps non-diffusible macromolecule. However, binding could also be an activation if the enzyme was fixed at a locus of action for a specific metabolic event. The possibility should also be kept in mind that the function of PME *in vivo* may not be as a hydrolytic enzyme but as a transmethylase.

The presence of invertase (13) and PME in the free space of *Avena* coleoptiles may indicate that there is a larger group of enzymes located in plant cell walls which are functional in cell wall metabolism and possibly in translocation and accumulation of solutes.

V. Summary

The pectin methylesterase of *Avena* coleoptiles is shown to be located in the free space. When coleoptiles are placed in dilute salt solutions some of the enzyme diffuses from the tissue. Estimations made from diffusion data and from fractionation in non-aqueous medium indicate that more than 90 % of the pectin methylesterase is adsorbed *in vivo*.

It is suggested that the cell wall may contain a group of enzymes functional in cell wall metabolism.

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The Effect of Glucose and Dinitrophenol on the Cyanide Inhibition of Oxygen Uptake in Wheat Root Tissue

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Introduction

It is known that many processes in the living cell are dependent on the presence of phosphate donors, the most important one being ATP. The inhibitory effect of 2,4-dinitrophenol (DNP) on processes like growth, syntheses of various cell components, and absorption has been successfully explained as a result of decreased ATP concentration in the cells due to inhibition of oxidative phosphorylation (Simon 1953, Laties 1957).

DNP in sufficiently high concentrations also inhibits oxygen uptake. In earlier investigations it was found that there are divergences in sensitivity to DNP inhibition of oxygen uptake between growing and mature root tissues (Eliasson and Mathiesen 1956) and between starving root tissue and tissue supplied with glucose (Eliasson 1959). It was maintained that these divergences could best be explained on the basis of the hypothesis that also DNP inhibition of oxidation is due to the decreased ATP concentration in the cells.

In this paper experiments regarding the cyanide inhibition of oxygen uptake in wheat roots are reported. Oxidase inhibitors like cyanide, which inhibit the oxidation mediated by the cytochrome system, also inhibit oxidative phosphorylation. The most important question of this investigation is whether the oxygen uptake in cyanide-inhibited tissue is affected by the low ATP concentration. If the supply of ATP is critical for respiration in such tissue, DNP even in low concentrations should be expected to increase the inhibition. Glucose when added to the roots is supposed to cause an increased conversion

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of ATP to ADP (Eliasson 1959). Normally this more rapid ATP consumption is balanced by a greater synthesis of ATP connected with an increased respiration. But if glucose is added to tissue where the oxidation is partially blocked by an oxidase inhibitor, it is possible that the concentration of ATP will be further lowered by the glucose.

The suitability of cyanide as a selective oxidase inhibitor has been questioned (Warburg 1946, James 1953), but the advantages of this substance over other inhibitors (cf. Robbie 1948) nevertheless have been considered to justify its use in this investigation. Since the time course of the respiration in the presence of cyanide is of particular importance for the problems investigated, great attention has been devoted to the technique for maintaining the cyanide concentration constant during the experiment.

Methods

The experimental material was roots of wheat seedlings grown in the dark under sterile conditions mainly according to earlier described methods (Eliasson 1955). After germination for two days on moist filter paper the seedlings were grown for three days in nutrient solution. The first developed root was then about 9 cm long. The part of this root from 4 to 9 cm above the tip was cut into 1 cm long pieces, and the oxygen uptake in these was determined according to the Warburg technique. In the Warburg flasks the root pieces were suspended in 0.02 *M* phosphate buffer, pH 6.7. When glucose and cyanide were used, these substances were added before the respiratory measurements were started. DNP was in some experiments added at the beginning of the experiment, in others it was added from the sidearm after readings for two hours.

For absorption of released carbon dioxide 15 per cent KOH solution was used in the flasks without cyanide. When the experimental solution contained cyanide, usually mixtures of $\text{Ca}(\text{CN})_2$ and $\text{Ca}(\text{OH})_2$ in concentrations stated by Robbie (1948) were used instead of KOH. In some of the experiments with low cyanide concentrations Robbie's mixtures of KCN and KOH were used. The cyanide concentration in the experimental solutions was controlled by analyses with Robbie's phenolphthalin method (Robbie 1944). The principle of this method is that cyanide oxidizes phenolphthalin to phenolphthalein in alkaline solution in the presence of Cu^{++} ions. The phenolphthalein formed is determined spectrophotometrically. This method was found to give too low values for solutions containing glucose, due to the fact that glucose reacts with cyanide in alkaline solution (Militzer 1949). For this reason the result of the analysis will be dependent on the time interval between the addition of alkali and the other reagents to the solutions. After some training, however, it is possible to obtain reasonably reproducible values also for solutions containing glucose (Table 1). DNP did not interfere with the cyanide analyses or influence the concentration of cyanide in the solutions. A considerable source of error in work with weak cyanide solutions was found to be the fact that cyanide rapidly disappears from solutions in contact with air (cf. Warburg 1946). Precautions to minimize this error were taken.

Table 1. *Disappearance of cyanide from solutions containing various quantities of wheat roots.* The roots were kept in 20 ml 0.01 M phosphate buffer, pH 6.7, in carefully closed Erlenmeyer flasks. Cyanide was added to a concentration of 2×10^{-4} M. Samples for cyanide determinations were taken immediately after cyanide addition and later at the times recorded. The values give the extinction of the analytic solution (2 ml experimental solution + 1 ml 0.4 per cent KOH + 1 ml reagent according to Robb) determined with a Beckman spectrophotometer at 5550 Å. 10^{-4} M cyanide gave the extinction 0.370.

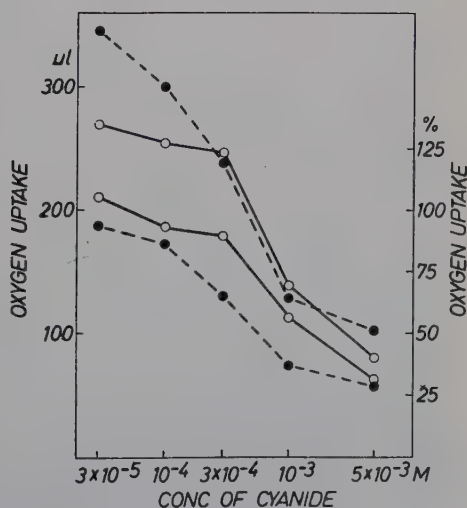
Quantity of roots (g)	Glucose (M)	Time in minutes after addition of cyanide				
		2	35	70	105	140
0	0	0.705	0.690	0.680	0.680	0.680
0	0.05	0.620	0.620	0.620	0.612	0.600
0.1	0	0.701	0.658	0.683	0.670	0.650
0.1	0.05	0.635	0.600	0.562	0.555	0.498
0.5	0	0.668	0.630	0.620	0.620	0.593
0.5	0.05	0.575	0.515	0.441	0.455	0.411
2.0	0	0.477	0.388	0.384	—	0.350
2.0	0.05	0.492	0.423	0.411	0.395	0.320

Results and Discussion

1. Effect of Exogenous Glucose on Cyanide Inhibition

It is often stated that cyanide inhibits the respiratory increase produced by glucose. This connexion between the effect of substrate supply and sensitivity to cyanide inhibition has been extensively treated by Commoner (1939, 1940). The concentration curves represented in Figure 1 are reasonably consistent with this classic picture of the connexion between cyanide inhibition and glucose effect. In the presence of glucose the oxygen uptake

Figure 1. *Effect of cyanide on oxygen uptake in wheat root tissue with and without glucose.* Cyanide was added about 25 minutes before the first reading. Oxygen uptake was determined for two hours. The two upper curves give the respiration in μ l oxygen per g fresh weight and hour and the curves below in per cent of the control. Solid lines: no glucose. Broken lines: 0.05 M glucose. Each point represents the mean of two parallels.



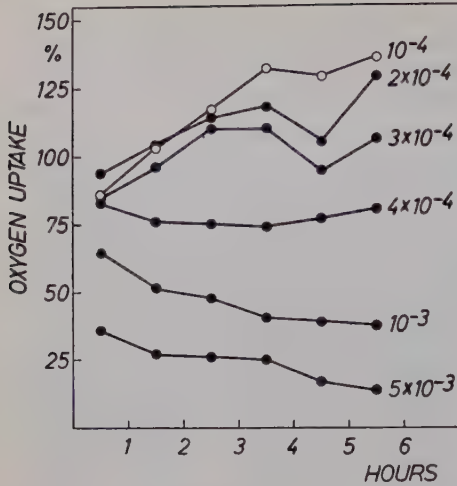


Figure 2.

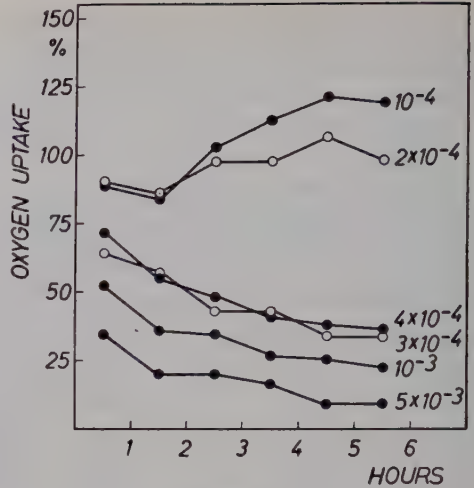


Figure 3.

Figure 2. Time course of oxygen uptake in the presence of cyanide without glucose. The figures to the right give the concentrations of cyanide (M). The experiments have been carried out in the same way as those of Table 2.

Figure 3. The time course of oxygen uptake in the presence of cyanide with $0.05 M$ glucose. Explanations cf. Figure 2 and Table 2.

shows a greater sensitivity to cyanide inhibition. The endogenous respiration is inhibited only by cyanide concentrations which cause a complete depression of the respiratory increase produced by glucose. This means that it is the cyanide-sensitive oxidation that is increased by glucose. It may be supposed that the oxidation capacity of the tissue is normally only partially used. Inhibition of the oxygen uptake is obtained only if enough cyanide is added to suppress the reserve capacity of the oxidation mechanism.

However, the picture is somewhat complicated if the time course of oxygen uptake in the presence of different concentrations of cyanide is considered. In Figures 2 and 3 the time curves determined at various cyanide concentrations with and without the addition of glucose have been put together. These curves show three interesting characteristics which will be further discussed: 1. Recovery or stimulation of oxygen uptake at low cyanide concentrations. 2. Progressively increased inhibition at high cyanide concentrations. 3. A remarkable increase in cyanide sensitivity caused by glucose at intermediary cyanide concentrations.

1. At low cyanide concentrations there is a stimulation of respiration over that of the control towards the end of the experiment in roots not supplied with glucose (Figure 2). However, the rate of oxygen uptake in the inhibited tissue is only insignificantly changed during the course of the experiment

Table 2. *Effect of cyanide on oxygen uptake in wheat roots with and without glucose addition.* In each Warburg flask about 0.1 g root tissue was suspended in 2 ml 0.02 M phosphate buffer, pH 6.7. Glucose was added immediately after excision of the roots, cyanide 25 minutes before the first reading. The values are averages for two parallel experiments and give oxygen uptake in $\mu\text{l O}_2$ per g fresh weight and hour.

Treatment	Time in hours after the first reading					
	0—2	2—4	4—6	0—2	2—4	4—6
	No glucose			0.05 M glucose		
<i>Experiment I</i>						
Control	271	225	199	348	339	338
10^{-4} M cyanide	255	280	263	300	365	405
<i>Experiment II</i>						
Control	275	216	212	398	389	365
3×10^{-4} M cyanide	246	238	212	240	165	124
<i>Experiment III</i>						
Control	313	248	211	410	376	370
10^{-3} M cyanide	183	110	80	184	116	88

(Table 2). Thus, this apparent stimulation is mainly due to the fact that the gradual decrease in respiration normally occurring in excised roots not supplied with exogenous substrate is prevented by cyanide. The stimulation is in this case not necessarily connected with an increased oxidase activity in the inhibited tissue during the experiment, but it may be due to the fact that cyanide disturbs the control mechanism which normally decreases the respiration in carbohydrate deficiency.

However, in the presence of glucose there is an increase in the rate of oxygen uptake during the course of the experiment at 10^{-4} M cyanide (Table 2) which must involve an increased oxidase activity. This increase may be explained as a result of formation of cyanide-insensitive oxidases in the presence of cyanide as discussed by Lundegårdh (1954). Another possibility is that the cyanide concentration decreases during the course of the experiment. In order to investigate this possibility, cyanide analyses of the experimental solutions were made after the respiratory measurements. In these a lower cyanide content was found in solutions with low initial cyanide concentrations than expected according to the connexion between cyanide concentrations in the center well and experimental solution given by Robbie (1948). Thus, the solutions with an initial cyanide concentration of 10^{-4} M with no glucose showed only about half this concentration when analyzed after the completion of the respiratory experiment. In solutions containing glucose still lower analytic values were obtained, but this discrepancy may be ascribed to reaction between cyanide and glucose during the analysis as

Table 3. *Effect of DNP and cyanide on oxygen uptake.* DNP and cyanide were added to the buffer solution before commencement of the determinations. No glucose was added. Oxygen uptake is given in $\mu\text{l O}_2$ per fresh weight and hour and in per cent of the control. Other details as in Table 2.

Cyanide (M)	DNP (M)	Time in hours after the first reading					
		0—2		2—4		4—6	
		$\mu\text{l O}_2$	%	$\mu\text{l O}_2$	%	$\mu\text{l O}_2$	%
<i>Experiment I</i>							
—	—	277	100	234	100	210	100
10^{-4}	—	260	94	290	124	281	134
3×10^{-4}	—	222	80	211	85	213	101
—	10^{-4}	527	190	453	194	362	172
10^{-4}	10^{-4}	210	76	220	94	235	112
3×10^{-4}	10^{-4}	162	58	103	44	77	37
<i>Experiment II</i>							
—	—	276	100	210	100	226	100
—	3×10^{-4}	441	160	383	182	273	121
10^{-4}	3×10^{-4}	147	53	82	39	61	27
3×10^{-4}	3×10^{-4}	91	33	54	26	40	18
1.5×10^{-3}	3×10^{-4}	75	27	42	20	33	15
<i>Experiment III</i>							
—	—	258	100	218	100	200	100
—	10^{-3}	94	36	57	26	39	20
3×10^{-4}	10^{-3}	75	29	61	28	36	18
10^{-3}	10^{-3}	73	28	66	30	36	18
5×10^{-3}	10^{-3}	34	13	27	12	4	2

already pointed out. At the higher cyanide concentrations used (10^{-3} and 5×10^{-3} M) a good coincidence was obtained between the analytic values and those expected.

When seeking an explanation of the fact that cyanide disappears from the most diluted solutions, some experiments were made with the purpose of investigating to what extent the roots absorb the cyanide present in the solution. The values from one of these experiments are recorded in Table 1. These experiments showed that cyanide absorption by the roots substantially decreased cyanide concentrations about 10^{-4} M. This absorption may be explained by the fact that cyanide reacts with various cell components (cf. Warburg 1946). As shown by the values of Table 1, the reaction between glucose and cyanide (Warburg 1946, Militzer 1949) is not of the same importance for cyanide disappearance at the pH used in this investigation. However, it must be pointed out that the experimental difficulties are great when working with such weak cyanide solutions. The only conclusion that can be drawn from these preliminary experiments is that a decrease in the cyanide concentration due to cyanide absorption by the roots is a contributing factor to the recovery of oxidation at low cyanide concentrations. Whe-

Table 4. *Effect of DNP addition to cyanide-inhibited roots.* DNP was added after respiratory determinations for two hours. Oxygen uptake is given in per cent of the control. Other details as in Table 2.

Cyanide (M)	DNP (M)	Time in hours after the first reading					
		0—2	2—4	4—6	0—2	2—4	4—6
		No glucose			0.05 M glucose		
10 ⁻⁴	—	95	125	133	87	108	120
10 ⁻⁴	5 × 10 ⁻⁵	92	125	145	83	96	120
3 × 10 ⁻⁴	—	90	110	101	60	42	34
3 × 10 ⁻⁴	10 ⁻⁴	80	90	58	61	43	29
10 ⁻³	—	58	44	38	45	31	24
10 ⁻³	10 ⁻⁴	57	45	33	47	34	21
5 × 10 ⁻³	—	31	26	15	28	19	9
5 × 10 ⁻³	10 ⁻⁴	35	26	17	26	18	9
—	10 ⁻³	100	81	41	—	—	—
10 ⁻⁴	10 ⁻³	94	67	29	—	—	—
3 × 10 ⁻⁴	10 ⁻³	89	45	24	—	—	—
10 ⁻³	10 ⁻³	59	37	22	—	—	—

ther the adaptation mechanism proposed by Lundegårdh is also of importance for this recovery is not revealed by these experiments.

2. At 10⁻³ M and 5 × 10⁻³ M cyanide the oxygen uptake is gradually decreased during the course of the experiment. As shown by the cyanide analyses this decrease is not caused by changes in cyanide concentration of the experimental solution, nor is there any reason to ascribe it to delayed penetration of cyanide into the tissue.

DNP in supra-optimal concentrations gives a similar time course of oxygen uptake (Table 3). This DNP effect may be explained as a result of ATP deficiency due to inhibition of phosphorylation (cf. Eliasson 1959). Oxidative phosphorylation is at least partly coupled to the cyanide-sensitive oxidation. Strong cyanide inhibition consequently causes a low ATP level in the cells. The progressive decrease of respiration in the presence of strongly inhibitory cyanide concentrations may thus be explained in the same way as the corresponding phenomenon caused by DNP, *viz.*, as a result of an inhibitory effect on oxidation by ATP deficiency.

3. The time course of oxygen uptake in the presence of 3 × 10⁻⁴ M cyanide is strongly affected by glucose (Figures 2 and 3, Table 2). The time-dependent increase of the inhibition in the presence of glucose may be explained by the fact that glucose in the cells is phosphorylated under conversion of ATP to ADP. Since the rate of ATP synthesis is limited by the cyanide inhibition, this increased ATP consumption will cause a greater deficiency of ATP in

tissue supplied with glucose than in starving tissue. This glucose effect thus is consistent with the above-mentioned hypothesis according to which ATP deficiency causes a time-dependent inhibition of oxidation.

2. *Interaction of DNP and Cyanide*

In Tables 3 and 4 the results from some experiments with DNP and cyanide have been collected. In the roots used in these experiments the maximal DNP stimulation was somewhat greater and the sensitivity to the inhibitory effect of DNP lower than in the roots used in an earlier investigation (Eliasson 1959). The reason for these divergences may be that in the present investigation only material from the main roots of the seedlings was used. Earlier the three first developed roots were used.

When DNP in concentrations which usually stimulate respiration was combined with low cyanide concentrations having in themselves only small effect on respiration, there was a stronger inhibition than that given by cyanide alone (Table 3). Especially when low concentrations of DNP were combined with 3×10^{-4} M cyanide, the additional inhibitory effect of DNP was strong. About the same degree of inhibition and the same time course of the residual oxygen uptake are obtained by 10^{-3} M cyanide, by 3×10^{-4} M cyanide combined with the same concentration of DNP or by 10^{-3} M DNP. A residual oxygen uptake of about 30 per cent seems to be rather resistant against the inhibitory effect of both DNP and cyanide (cf. Commoner 1940). This residual respiration decreases during the course of the experiment.

These experimental results may be explained on the basis of the hypothesis that ATP deficiency inhibits the oxidation processes. An important characteristic of this hypothetical "ATP deficiency inhibition" is that it develops gradually. DNP is known to decrease the concentration of ATP in the cell. At low DNP concentrations this decrease is not serious enough to have deleterious effects on the oxidative mechanism. For this reason the increase in ADP concentration or the uncoupling effect of DNP causes a respiratory stimulation. Cyanide in a concentration of 3×10^{-4} M may be supposed to cause a certain inhibition of the oxidative phosphorylation but this inhibition is not sufficiently strong to cause an ATP deficiency severe enough to give the characteristic time-dependent decrease of the oxygen uptake. In this case the inhibition of oxygen uptake is produced only by the blocking of oxidase molecules by cyanide. If, however, the weak inhibitory effect of 3×10^{-4} M cyanide on oxidative phosphorylation is combined with the inhibitory effect of low DNP concentrations on the same process, the result will be an ATP deficiency severe enough to affect the oxidation.

Summary

The effect of cyanide on the oxygen uptake of wheat root tissue has been investigated. Especially the time course of the inhibition during an experimental period of six hours was studied. At low cyanide concentrations (about 10^{-4} M) the inhibition was decreased during the course of the experiment, and towards the end of the experiments oxygen uptake in the presence of cyanide was higher than in the control. At cyanide concentrations about 10^{-3} M the inhibition increased during the experiment.

The possibility that these time-dependent changes in inhibition were caused by changes in the cyanide concentration of the experimental solutions was investigated by determination of the cyanide concentrations after the respiratory determinations. At low cyanide concentrations the analyses showed that the concentration decreased during the experiment. This decrease is due at least partly to the fact that the root tissue absorbs cyanide.

Glucose increased the sensitivity of the roots to cyanide when added to the solution to a concentration of 0.05 M. This may be partly interpreted as a consequence of the fact that glucose causes a more efficient use of the oxidative capacity of the cyanide sensitive oxidases. However, at cyanide concentrations about 3×10^{-4} M, glucose causes an increase of the cyanide inhibition by inducing a time-dependent decrease in the oxygen uptake. This effect is interpreted as a consequence of a decreased level of phosphate donors (ATP) caused by glucose.

The interaction between cyanide and 2,4-dinitrophenol (DNP) was investigated. DNP in concentrations normally giving a respiratory stimulation increased the inhibition caused by low cyanide concentrations. The experiments support the hypothesis that deficiency of ATP in the cells causes a gradually increasing inhibition of the oxidation processes.

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The Effect of Carbon Dioxide on Hydroactive Closure of the Stomatal Cells

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The investigations of Linsbauer (1917), Sayre (1926) and Scarth (1929, 1932) on the effect of carbon dioxide on the movements of the stomatal cells have been followed by numerous publications on this subject (*cf.* Stålfelt 1956, p. 410). That use has chiefly been made of knowledge of the carbon dioxide effect to explain the photic processes of the stomatal mechanism, *i.e.*, photoactive opening and closing, must presumably be ascribed to the basic assumption that the CO_2 content of the cells — or at any rate the pH of the cellular contents — is altered by photosynthesis.

It may perhaps seem less well motivated to seek a relation between CO_2 and the closing movements of the stomatal cells elicited by a water deficit, namely, the hydroactive closing movements. However, this question was also taken up by Scarth and Shaw (1951b) who, on the basis of their studies, expressed the view that “accumulation of CO_2 is an intermediate factor between wilting and stomatal closure”. These authors interpreted the cause of closure to be an inhibition of photosynthesis produced by the water deficit, with a resulting increase in the CO_2 content of the cells.

Moreover, in their attempts to explain the stomatal movements, Scarth and Shaw (1951) — and later Williams (1954) as well — put forward the hypothesis of a contribution by processes of an adenoid nature. Further evidence in this direction is provided by my earlier investigations on the mode of action of the water deficit (Stålfelt 1957). They indicated that the hydroactive closing reaction is dependent partly on a relatively slow decrease in

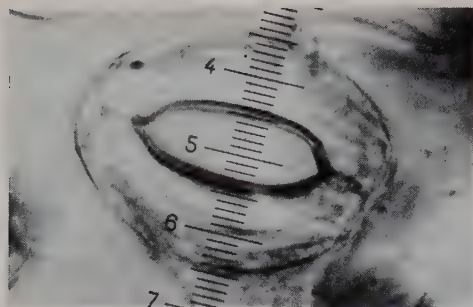


Figure 1. *Stomatal measurements in leaf of Vicia faba*. A piece of leaf 5×10 mm (*n.b.* not a surface section) is placed in liquid paraffin on an object glass covered by a cover slip. The width of the guard cells and opening width are measured with an ocular micrometer, the light passing through the object. Leitz Dialux Planoptik microscope (plano objectives). Dry objective 40 X. Ocular periplane GF 25 with scale. 1 scale division = 1.5μ . Photo: Bo Kuylenstierna.

the osmotic value of the guard cells, and partly on a more rapid process, namely, an adenoid form of water output. The latter process, *i.e.*, the water secretion, is sensitive to changes in the carbon dioxide pressure; it is favoured by CO_2 , which thus promotes closure (Stålfelt 1957). This is a prerequisite for the investigations reported in the present paper.

Methods

For measurement of the stomatal width, use was made of a Leitz Dialux microscope with plano objectives. With this instrument, the stomatal cells in whole leaves and pieces of leaves can be measured even without immersion (Figure 1). As in earlier investigations, the guard cells were measured in rectangular pieces of leaf 5×10 mm in size; the mean value of 10 measurements in each sample was taken.

If the standard deviation of the single measurements and of the mean values is calculated, it is generally found to increase with stomatal width. It is, in fact, smallest when the stomata start to open, and increases in size as opening proceeds. One of the reasons underlying these changes is that all the stomata in a single leaf do not have the same ability to open; certain stomata have a greater opening maximum than others. In leaves of *Vicia faba*, the standard deviation of the individual stomatal values was initially $\pm 0.4 \mu$, but increased to $\pm 2.6 \mu$ when opening had reached a value of 10μ . The standard deviation of the corresponding mean value was ± 0.1 and $\pm 0.9 \mu$, respectively. The figures given in the tables and diagrams are the mean values.

The standard deviation is also dependent on the previous history and pretreatment of the leaf. The greater the water deficit to which the plant has been exposed, the greater is the standard deviation. The function of the guard cells is more or less arrested by a high water deficit (Iljin 1922). These difficulties can be avoided, and a uniform, regularly reacting experimental material can be obtained by an adequate supply of water during cultivation, or by choosing an object that has not suffered from any great water deficit.

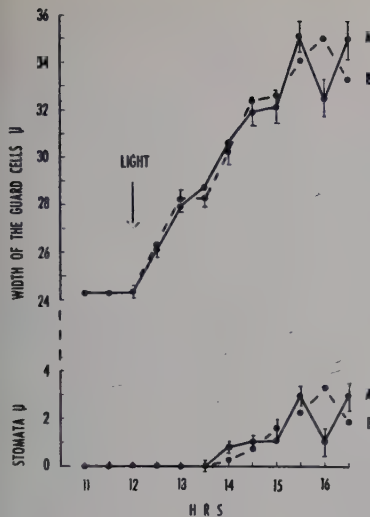


Figure 2.

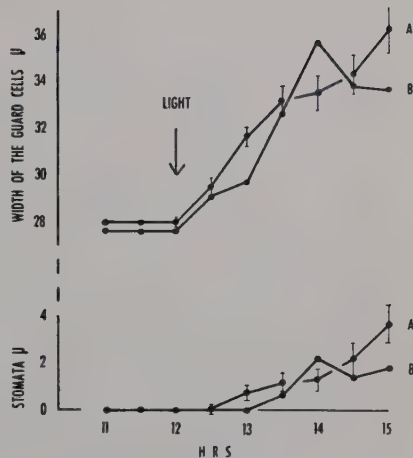


Figure 3.

Figure 2. Photoactive opening movements of stomatal cells on the upper side of a leaf floating on water with lower side downwards (A), and on a leaf with only the stalk in water (B). — The opening movement is about as strong in both cases. Light intensity: 32,000 lux. Temperature: 22–24°C. *Vicia faba*.

Figure 3. Photoactive opening movements of the stomatal cells (on upper side of leaf) in pieces of leaf (1×2 cm) floating on water with the lower side downwards (A) and in pieces of leaf immersed below the surface of the water (B). — The opening movement is as strong in both cases. Otherwise as in Figure 2.

Experimental

A. Relation Between the Photic Opening Movements and Gas Exchange in the Leaves

The experimental object consisted chiefly of *Vicia faba*, grown in the open with a good supply of water. In each measuring series, a pair of leaflets was taken from the same leaf, *i.e.*, the two opposite leaflets on a basal leaf of the plant. One was used as the experimental object and the other as the control. *Rumex sativa* was used in some experiments. Before starting the experiments, the severed leaves had stood in darkness and saturated moisture for 12–14 hours.

a. *Leaves floating on water.* The photic stomatal opening was compared in leaves floating on water (lower side downwards) and in leaves with only the leaf stalk standing in water. The intensity of light was 32,000 lux and the temperature 22–24°C. Pieces were excised from the leaflets at fixed intervals and examined microscopically.

It was found that the stomata opened at the same rate in the floating leaf as in the control one. The only difference was that the course was generally evenner and more regular in the floating leaf. In five experimental series the opening movements were followed for 3 hours. The increase in width of the guard cells during this time was a mean $5.4\ \mu$ in the experimental leaves and $5.6\ \mu$ in the controls. The mean value of the opening width was $2.0\ \mu$ and $1.4\ \mu$, respectively. Thus, arrest of the gas exchange on the lower side of the leaf did not prevent the photoactive opening process.

b. *Leaves immersed in water.* Scarth, Whyte and Brown (1933) showed that the stomata open even in leaves immersed in water or oil and illuminated. The result was the same in my experiments with *Vicia faba* (Figure 3). In leaves immersed in water, the stomatal opening movements were characterized by an even course. This is presumably to be ascribed to the fact that, in such objects, photic opening is not disturbed by the hydroactive closing processes; the turgor is high in both immersed and floating leaves, and no water deficit develops. If, on the contrary, the leaves are exposed to a water deficit, photic opening is disturbed.

Figure 4 shows an experiment including a leaf that had been allowed to transpire freely (with only the stalk in water) and that had lost water during the experiment. In this leaf, photoactive opening was disturbed first by addition of the effect of passive opening occurring at a deficit of 1—2 per cent. Later, opening was also disturbed by the fact that the deficit initiated a hydroactive closing reaction, which resulted in regression of the opening reaction for 0.5—1 hour.

c. *Effect of wound damage.* If the leaf is cut into pieces, e.g. 15×20 mm, and the pieces are placed so that they float on water and are exposed to light, the stomata open photoactively in the same way and at the same rate as in whole leaves floating on water. In experiments of this kind, the objects were exposed to 32,000 lux for 3 hours at $22\text{--}24^\circ\text{C}$. The following mean values were obtained in 20 measurements. The width of the guard cells increased by a mean $11.0\ \mu$ in both the cut experimental leaves and the controls (whole leaves). The stomatal width increased by $2.9\ \mu$ in the experimental leaves and by $3.0\ \mu$ in the controls. Thus, wound damage to the pieces of leaf did not alter the course of the stomatal movements.

d. *Liquid paraffin as medium.* If the experimental conditions are altered by substituting liquid paraffin for water, the photic opening reaction is essentially unchanged. Even in pieces of leaf immersed in paraffin and exposed to light, the photoactive opening process was found to have a largely regular course for several hours (Figure 5). This is of particular importance from the point of view of experimental technique, since it opens up a new means of studying the effect of a water deficit on the hydroactive phase of stomatal closure.

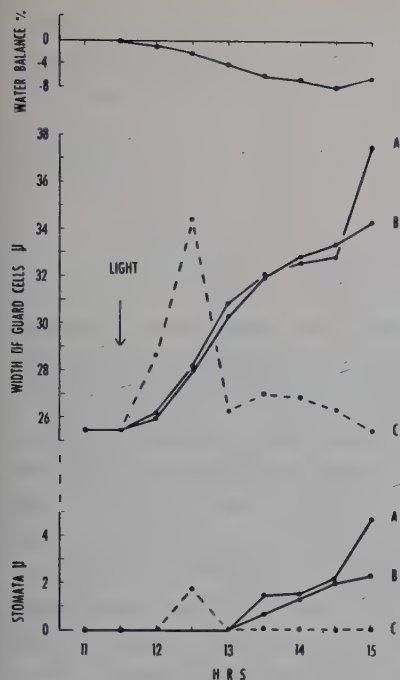


Figure 4.

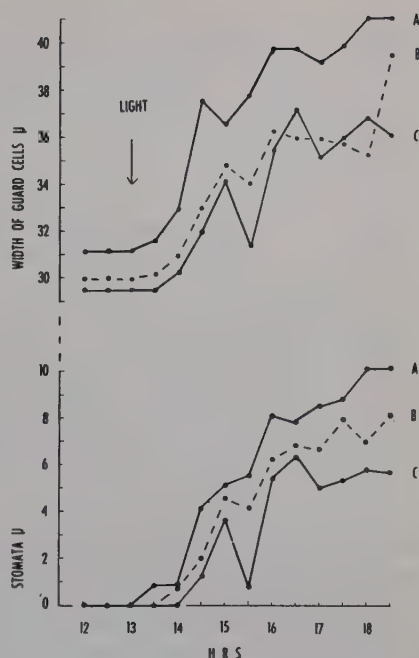


Figure 5.

Figure 4. Photoactive opening movements of the stomatal cells in pieces of leaf (1×2 cm) floating on water (A), in pieces of leaf immersed below the surface of the water (B), and in a whole leaf exposed with only the stalk in water, and otherwise allowed to transpire freely (C). — In leaf C, a water deficit arises of the size shown in the uppermost diagram. When the deficit has reached 2–4 % (calculated on the fresh weight at water saturation), a hydroactive closing movement occurs. Otherwise as in Figure 2.

Figure 5. Photoactive opening movements of the stomatal cells in pieces of leaf immersed in liquid paraffin. Three water-saturated leaves (A, B, C), kept in darkness and moisture-saturated air for 14 hours, were cut into pieces (1×2 cm), which were immersed in paraffin and exposed to light. Otherwise as in Figure 2.

It can be presumed that no appreciable water exchange takes place in paraffin. Consequently, this should make it possible to keep the object at the same deficit for a long time, which cannot be done if the surrounding medium is air or water.

The experiments have thus shown that photoactive opening — i.e., the photic opening process which occurs without being disturbed by hydroactive closing reactions — takes place independently of the gas exchange in the leaf, namely, independently of an exogenous supply of carbon dioxide and oxygen. Naturally, this does not imply that the photic processes are indepen-

dent of oxygen and carbon dioxide. It is rather that a respiratory-photosynthetic system deprived of air and water can be envisaged as "self-supporting" for some time with respect to oxygen and CO_2 . This is because photosynthesis receives CO_2 from respiration, and respiration receives O_2 from photosynthesis.

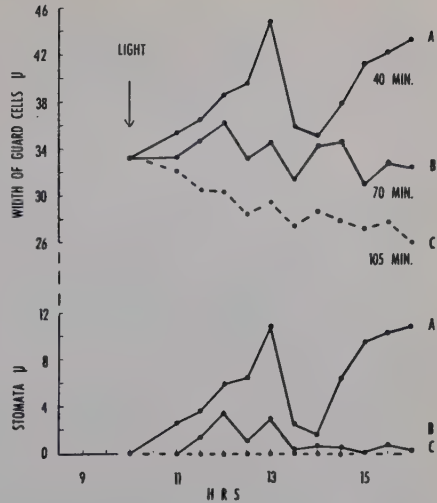
B. Relation Between Hydroactive Closing Movements and Gas Exchange in the Leaves

It has been shown earlier that the water deficit produces closing movements in the stomatal cells, and that the deficit then requires a certain time to take effect (Stålfelt 1929). This period can be denoted as the "reaction time" of the deficit; not until it is ended does the closing movement start. It was also shown that the size of the closing movement increases with the duration of action of the deficit, that it is initiated only when the deficit has exceeded a certain minimum value — the threshold value — and that the closing movement then proceeds to the same degree as the deficit increases (Stålfelt 1929, 1932). To avoid misunderstanding, it must be emphasized that the term "threshold value" denotes the lowest active deficit, *i.e.*, the deficit at which the movement starts, and not the deficit at which closure is completed (Stålfelt 1929, p. 308). The latter may vary, since closure is dependent not only on the size of the deficit, but also on its duration of action and on the prevailing light intensity.

The hydroactive closing movements have been studied earlier in leaves in air, *i.e.*, leaves with free exchange of CO_2 , O_2 and water vapour. Since photic opening of the stomatal cells has now been found to occur even in objects in which the gas exchange is arrested, it should be possible to study the hydroactive closing movements under these conditions as well.

a. *Water deficit of same size but different duration of action.* The experiment recorded in Figure 6 can be taken as an example. The object was a leaf of *Vicia faba* which had stood in darkness and saturated moisture for 14 hours. One of the leaves (C) was allowed to transpire in darkness without supply of water until its weight loss was 10 per cent of the fresh weight of the leaf; this took 35 minutes. The leaf was then returned to darkness and saturated moisture, still without any water supply, for 70 minutes. After this time, *i.e.*, after 105 minutes counted from the beginning of transpiration, the leaf was cut in pieces 10×20 mm, immersed in liquid paraffin and exposed to light. During this time, two other leaves treated in the same way were exposed to light, so that their deficit was also 10 per cent. In these cases, however, the treatment started later, the time between beginning of transpiration and exposure to light therefore being only 70 and 40 minutes, respectively.

Figure 6. Experiment as in Figure 5, except that the leaves had a water deficit of 10 % (a water loss corresponding to 10 % of the fresh weight at water saturation) when they were exposed to light. The duration of action of the water deficit (time between beginning of transpiration and exposure to light) differed; in leaf A it was 40 minutes, in B 70 minutes, and in C 105 minutes. Otherwise as in Figure 2. — Thus, in this experiment, the size of the deficit is the same, but its duration of action differs. The longer the duration, the stronger is the hydroactive closing reaction.



At intervals of 30 minutes, a piece was taken from each leaf and examined under the microscope.

The results, recorded in Figure 6, show that the photoactive opening reaction has started in leaf A; its water deficit, which had acted for only 40 minutes on starting exposure, has not yet elicited closing movements. The onset takes place later, and cell width and opening width are then characterized by the changes dependent on the antagonism between photic opening and hydroactive closure. As a result, sometimes one reaction is predominant, and sometimes the other. The course differs in leaf B. When this leaf was exposed to light, the deficit had already initiated closing movements. Photic opening was therefore smaller than in A, but in B as well, opening is characterized by the unevenness based on the antagonism between the opening and closing processes. The course is, however, more even in B than in A. In leaf C, the deviation from leaf A is still greater. When light exposure starts, the effects of the deficit have reached the stage where the photic opening movements are entirely inhibited. Instead of taking up water, the guard cells give off water, so that the width of the stomatal apparatus decreases.

The experiments show that arrest of the gas exchange of the leaf does not prevent hydroactive closure. Leaves in paraffin react in the same way as leaves in air, and the strength of closure is dependent on the duration of the deficit. Furthermore, the experiments show that CO₂ deficiency does not lead to opening of the guard cells in light, when the hydroactive closing reaction has reached sufficient intensity.

b. Water deficit of different size but same duration of action. In the experi-

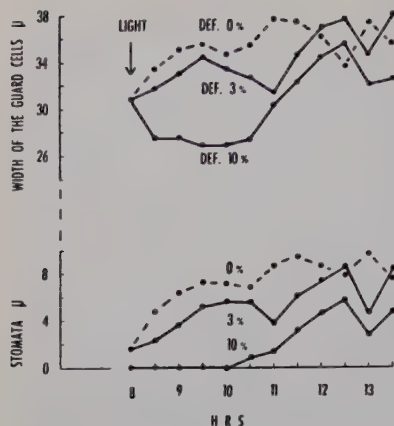


Figure 7.

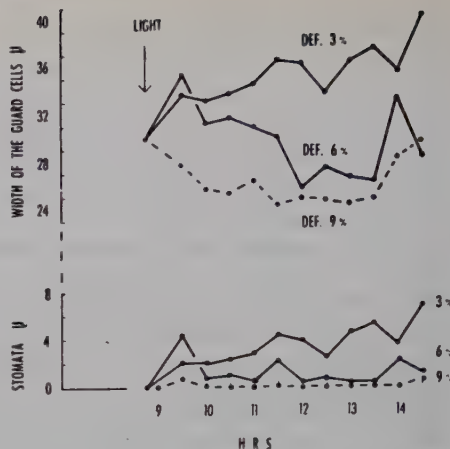


Figure 8.

Figure 7. Experiment as in Figure 5, except that the three leaves had a water deficit of 0, 3 and 10 % when they were exposed to light. The duration of action of the deficit (time between beginning of transpiration and exposure to light) was the same in all three cases, i.e., 60 minutes. *Rumex sativa*. Otherwise as in Figure 2. — Thus, in this experiment the duration of action of the deficit is the same, but its size differs. The greater the deficit, the stronger is the hydroactive closing reaction. The difference is, however, apparent only in the first 3 hours: thereafter, the reaction of the leaves is more similar. This is presumably due to inhibition of the gas exchange caused by the liquid paraffin.

Figure 8. Experiment as in Figure 7, except that the water deficit was 3, 6 and 9 %, and its duration of action was 90 minutes in all three leaves. *Vicia faba*.

ment recorded in Figure 7, the water deficit of the leaves differed (0, 3 and 10 per cent) but the duration of action of the deficit (time between beginning of transpiration and exposure to light) was the same (60 minutes). The object was *Rumex sativa*. Figure 8 shows an experiment with a water deficit of 3, 6 and 9 per cent. The object was *Vicia faba*.

It is apparent from both experiments that photic opening decreases when the deficit increases, and that opening is finally prevented altogether. When the deficit has reached high values, the water output of the stomatal cells becomes so great that the width of the guard cells becomes less than that before exposure of the object to light. The course is thus the same as in objects with free gas exchange.

The effect of the deficit is, however, relatively brief in objects immersed in paraffin. In this respect, the leaves in oil differ from leaves surrounded by air. Thus, after a few hours, hydroactive closure ceased in the leaves immersed in paraffin. In the experiment in Figure 7 (*Rumex sativa*), this occurred already after 2—3 hours, and in that shown in Figure 8 (*Vicia faba*)

after 4—5 hours. The changes are possibly related to the “self-support” with respect to O₂ and CO₂ mentioned earlier (p. 696), and which probably cannot maintain these functions for more than a short time.

C. *Effect of Carbon Dioxide*

a. *CO₂-free medium.* Experiments of the kind illustrated in Figures 6, 7 and 8 show that inhibition of an exogenous supply of O₂ and CO₂ does not prevent the hydroactive closing reaction from coming into effect, and that such arrest of CO₂ does not produce stomatal opening in light, if the leaf has lost a certain quantity of water.

b. *Administration of CO₂.* Leaves that had stood in darkness and saturated moisture for 12—14 hours were allowed to transpire without water supply until their water loss had reached a certain value. They were then returned to darkness and saturated moisture, still without any water supply. When sufficient time had elapsed (at least 60 minutes) that hydroactive closure could be expected to have started — the water deficit was then unchanged — the leaves were cut into pieces 5×10 mm, and placed on moistened filter paper in glass tubes, which were corked. The tubes were divided into three series; in one series CO₂-free air was passed through the tubes, and in the other two series air containing CO₂. All the tubes were exposed simultaneously to light; every 30 minutes a tube was taken from each of the series, and the pieces of leaf examined under the microscope.

Examples of measurements of this kind are shown in Figures 9 and 10. The water deficit of the leaves — which in both cases was initially 4 per cent — had probably increased during illumination, since, even in leaves placed in a moisture-saturated chamber, some transpiration occurs on exposure to light. It is apparent from the experiments that the deficit elicited hydroactive closure, which was manifested either as a decrease in width and opening of the guard cells, or as weaker photoactive opening. Closure was observed in all three series, and was most marked in the leaves supplied with CO₂.

Experiments of this kind were repeated several times with varying water deficits (4—9 per cent). In each case, the effects of two concentrations of CO₂ were compared with those of CO₂-free air. Three such measuring series comprised one experimental group. With *Vicia faba* as the object, 7 such groups of experiments were made, the CO₂ concentration being 0, 0.03 and 0.5 per cent. During exposure to light, lasting for 3—4 hours, the width of the stomatal apparatus and the opening width were measured.

The mean values showed that the opening movement was smaller in the samples surrounded by CO₂-containing air than in the samples in CO₂-free

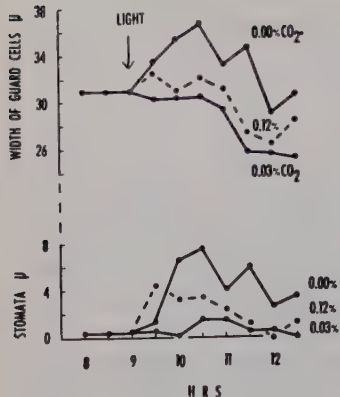


Figure 9.

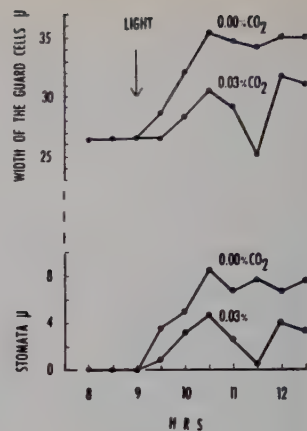


Figure 10.

Figure 9. *Promotion of the hydroactive closing by CO₂.* Pieces (1×2 cm) of turgescient leaves, kept in darkness and moisture-saturated air for 14 hours, were allowed to transpire until the water deficit had reached 4 %. After 80 minutes (counted from beginning of transpiration) the pieces of leaf were put in glass tubes (15 ml), containing filter paper moistened with water. Air containing 0, 0.03 and 0.12 % CO₂ was passed through the tubes, which were corked and exposed to light. — The hydroactive closing reaction, which prevents photic opening, is promoted by CO₂. *Vicia faba*.

Figure 10. *Experiment as in Figure 9. Rumex sativa.*

air. In the samples that had lain in air containing 0.03 per cent CO₂, the width of the stomatal apparatus was 3.6 μ less than in the control lying in CO₂-free air. The corresponding value in the sample placed in 0.5 per cent CO₂ was 3.4 μ less than in the control. Three groups of experiments of the same kind were made with *Rumex sativa*. The CO₂ concentration was 0.03 and 0.12 per cent, and in both cases resulted in a value for stomatal width that was 3.4 μ less than that of the control (0.00 %). These values are in agreement with the findings of Freudenberg (1941) and Heath (1949, 1950) that the stomata responded to changes in the carbon dioxide content of the air below the normal 0.03 per cent.

The experiments thus show that hydroactive closure occurs even when the exogenous supply of CO₂ is cut off. Moreover, this closure also takes place in light, and it is appreciably increased by a supply of CO₂.

It was demonstrated in an earlier investigation that the effect of carbon dioxide probably consists of a rise in the water secretion of the guard cells (Stålfelt 1957). Through this effect, CO₂ is of importance for stomatal opening as well, since the stomatal movements are determined by the balance between the processes of opening and closure. If, for example, CO₂ is removed,

the water secretion of the guard cells decreases and, for this reason, the rise in turgor produced by light is more rapid, *i.e.*, photic opening is promoted. Administration of CO₂ has the opposite effect, in that the secretion is increased, so that the rise in turgor takes place more slowly or is prevented. In this way, the direct effect of CO₂ on the movements of the guard cells results in an indirect effect on their opening. *Its effect on the opening and closing movements can thus be traced to the same mechanism, namely, the action of carbon dioxide on the hydroactive closing movement.*

Consequently, if CO₂ partakes in the normal stomatal movements, this can occur in such a way that it promotes or inhibits the reaction-compelling processes, *i.e.*, the osmotic water uptake and water output of the cells. It is therefore also conceivable that, in this interplay of forces, carbon dioxide has a role as an eliciting and regulating factor.

Discussion

In the hydroactive closing movements of the stomatal cells, two kinds of movement process can be distinguished (Williams 1954, Stålfelt 1957):

1. A fairly rapid closing movement, which is caused by a probably non-osmotic water output, *i.e.*, a water secretion. As stated earlier, this reaction is sensitive to CO₂ (*cf.* p. 692). The results reported here, showing that hydroactive closure is promoted by CO₂, can be explained by the theory of the water secretion of the guard cells, and the promoting effect of CO₂ on this reaction.

2. A decrease in the osmotic value of the guard cells which occurs relatively slowly, and which leads to complete closure and to stabilization of the closure.

The way in which the water deficit elicits these reactions is not known. A suggestion for solution of the problem has been given by the investigations made in recent years on the sensitivity of the stomatal cells to carbon dioxide (Scarth 1932, Scarth, Whyte and Brown 1933, Scarth and Shaw 1951 a and b, Freudenberger 1941, Heath and Milthorpe 1950). It is on the basis of these investigations that Scarth and Shaw (1951 b) put forward the hypothesis of CO₂ as the connecting link between the water deficit and the guard cell movements. This hypothesis is supported by the experiments reported in the foregoing, which showed that it is, in fact, the hydroactive closing movement that is the CO₂-sensitive phase. Since it is also known that both photosynthesis and respiration — and thus CO₂ metabolism as well — are influenced by the water content of the cells, this hypothesis seems to have a sound factual basis.

If, however, we scrutinize the known facts about the relation between the water content of the cells on the one hand, and photosynthesis and respiration on the other hand, we must admit that the hypothesis still lacks one important prerequisite. It has not yet been established that the slight water deficits which elicit stomatal closure, and which often amount to only a few per cent of the water content of the leaf, also influence photosynthesis and respiration.

Photosynthesis is known to be sensitive to the state of hydration of the plant in many cases. It has, for example, been found that photosynthesis decreases with a supply of water to the plant and with a previous water loss, that it is dependent on wilting, and therefore diminishes when the object suffers a water loss through transpiration, or by placing in a hypertonic solution. But such experiments merely show that the water deficit — in the degree to which it has been ascertained — inhibits photosynthesis only when high values are reached. As far as the cause of hydroactive closure is concerned, it is not, however, the high deficits which are decisive, but the deficits of a few per cent which initiate closure. Only when these deficits are measured can it be determined whether inhibition of assimilation is a completely reversible process — produced, for example, by stomatal closure — or whether it is a result of more or less reversible destruction of the plasma caused by the water loss.

Pisek and Winkler (1956), when studying this question, made direct measurements of the water deficit and stomatal width, and put these values in relation to photosynthesis. Their results provide no evidence in support of the view that photosynthesis is inhibited by a water loss, as long as the stomata are open. In the objects in question (*Pulmonaria*, *Asarum*), the sensitivity to the water deficit was greater in the stomatal apparatus than in photosynthesis; the CO_2 uptake ceased concurrently with hydroactive closure of the stomata, or in any event shortly after it.

If, however, it could be shown that CO_2 contributes to photic opening, this would imply that opening — like closure — is under dual control and has two causes, *i.e.*, both the osmotic water uptake of the guard cells, and the inhibition of secretion from the guard cells caused by decreased CO_2 pressure. The role of carbon dioxide would then consist of facilitating and hastening the process, and perhaps also of controlling the motive force. The osmotic water uptake of the cells acts as the motive force, since it rises concurrently with photic opening (Stålfelt 1955).

A change in the CO_2 concentration of the cells should also be able to result from a change in the intensity of *respiration*. Since it is known that respiration may also be influenced by the water deficit, CO_2 would be able in this way to participate in the causal relation between deficit and stomatal closure.

This would explain, for example, the results presented in Figures 6—8

in the present paper. In these experiments, the deficit is the cause of stomatal closure (or an impediment to photic opening), even when it is allowed to act in darkness (time before the exposure to light). The relation could be explained by the assumption that the deficit increased the production of respiratory CO₂; only when the CO₂ had accumulated for a certain time (more than 40 minutes in the experiment in Figure 6) would the concentration be sufficient to inhibit photic opening.

However, in this case as well, the same important prerequisite is lacking as in the former case: it has not been shown that slight deficits in general produce an increase in the intensity of respiration. Respiration is known to be altered by a high water deficit (for literature, see Stocker 1956); for this reason, it can both increase and decrease. It increases with increased water deficit, *e.g.* owing to diminished moisture of the soil, or a fall in the ground water. It also increases when the leaf wilts (water loss of *e.g.* 29 or 37 per cent; Iljin 1923) and after wilting, which always presupposes a large deficit; on the other hand, respiration has also been stated to decrease when the deficit increases (for references, see Stocker 1956).

Data regarding the effect of lower deficits are, on the contrary, sparse. Mothes (1931) found that respiration increased with a water deficit from 0 to 10 per cent, but only in certain species. Experiments with tobacco plants showed that a rising deficit from 0 per cent upwards produced an increase in respiration in some leaves, but a decrease in others. In *Phaseolus vulgaris*, respiration was dependent on a high deficit (30 per cent), but not on lower ones (0—10 per cent). It was also dependent on the nature and age of the organ.

Pisek and Winkler (1956) could not establish any relation between respiration and the lower water deficits. Thus, respiration underwent no change when the water deficit of the leaf rose from 2 to 12 per cent (*Betula*) or from 9 to 18 per cent (*Asarum*). A rise did not occur until the deficit started to approach sublethal values.

It can therefore scarcely be asserted that the investigations hitherto reported on the relation between the water deficit of stomata-bearing organs and gas exchange do, in fact, lend support to the view that such deficits which initiate stomatal closure would inhibit photosynthesis, or stimulate respiration. Consequently, in order to answer this question, more detailed investigations must be made on the CO₂ content of the cells and its changes with low water deficits or, preferably, on the pH of the cells and low deficits.

Additional factors to the altered CO₂ values may be responsible for the changes in pH. It is conceivable that the deficit acts on those parts of the metabolism which produce or utilize other acid substances than CO₂. Such processes are known to cause variations, even diurnal periodic variations, in

the cell sap of Crassulaceans and other succulents (*e.g.* Sideris 1948, Bruinsma 1958). Similar variations, even if on a smaller scale, may perhaps occur in stomata-bearing plants as a whole. It does not necessarily follow from the fact that the CO_2 content of the leaf varies, and that it affects the stomatal movements, that it elicits these movements under normal conditions. Its effect on the guard cells can be interpreted as a special case of the effect of acid substances in general.

Summary

The sensitivity of the hydroactive closing reaction to carbon dioxide has been investigated in leaves of *Vicia faba* and *Rumex sativa*. The width of the guard cells and of stomatal opening was measured microscopically in pieces of leaves (*i.e.*, not in sections). The experiments led to the following results and conclusions:

A. In leaves immersed in liquid paraffin, hydroactive closure takes place normally. It is not prevented by inhibition of the leaf's exchange of O_2 and CO_2 , and it occurs in both light and darkness. Even in leaves immersed in paraffin, closure is dependent on the size and duration of action of the water deficit.

Inhibition of the CO_2 supply does not lead to stomatal opening in light if the hydroactive closing reaction has reached sufficient strength, *i.e.*, if the water deficit is large enough or has acted for long enough. Photic opening decreases when the deficit increases, and fails to occur when the deficit has exceeded certain values.

B. Supply of carbon dioxide increases hydroactive closure. The reaction presumably consists of an increase in water secretion of the guard cells.

Since a balance exists between the photic opening reactions and the hydroactive closing reactions, photic opening is prevented when the CO_2 pressure rises. The inhibitory effect of CO_2 on photic opening of the stomatal cells is thus of an indirect nature; it is, in fact, a result of a closing reaction being promoted by CO_2 .

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The Acidic Growth Inhibitor of Potato Tubers in Relation to their Dormancy

By

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Introduction

Dormancy or rest in potato tubers refers to that condition in the tuber immediately after harvest when sprouting does not occur. Some authors distinguish between rest and dormancy, rest being the period when the tuber is incapable of sprouting even under favorable conditions, and dormancy being merely lack of visible sprout growth, whether because of the resting state of the tuber or because of unfavorable storage conditions (Stuart and Milstead, 1934). However, these terms, rest and dormancy, are often used interchangeably and it is not at all clear that such a distinction has any real meaning. Rosa (1928) reported that primordia of the vegetative sprouts developed during the later stages of tuber growth as well as during the dormant period after harvest and that the rate is merely accelerated at the end of rest. At temperatures favorable to growth, he felt that the meristematic region is probably never entirely inactive. Davidson (1958) also made anatomical studies of the bud and found that the sprout grew from 0.2 mm. at harvest time to 0.45 mm. at the stage when it first became visible. Like Rosa, he felt that rest and dormancy were not two distinct states. In this discussion, both dormancy and rest will be used interchangeably to designate that period after harvest when there is no visible sign of sprout development.

Hemberg (1946) reported that potatoes contain an ether-soluble, acidic substance which causes inhibition of oat coleoptile curvature. This substance he found to be present in the peel of freshly harvested potatoes but absent in

the peel of potatoes which had been stored for some time. Hemberg suggested that it is the presence of this substance, apparently identical with the β -inhibitor of Kefford (1955), which is primarily responsible for the failure of potato buds to sprout immediately after harvest.

Blommaert (1954) and Varga and Ferenczy (1957 a), using paper chromatographic separation of the acidic inhibitor and straight growth assays, also reported that the inhibitor decreased in activity during storage of the tubers. However, Burton (1956), using a different paper chromatographic separation, found that the amount of acidic inhibitor in potato peels fluctuated during the storage period, but that onset of sprouting did not necessarily coincide with disappearance of the inhibitor.

The bioassays used to test for inhibition were coleoptile curvature (Hemberg 1946, 1949), coleoptile straight growth, (Blommaert 1954, Burton 1956, Varga and Ferenczy 1957 a, Hemberg 1958) and growth of adventitious roots on potato sprouts (Burton 1956). In only one case was the effect of the inhibitor tested on potato buds themselves (Hemberg 1949). One-eyed pieces of potato, about three-fourths inch in diameter, were soaked in extracts of peel of dormant potatoes. The extracts had no effect in hastening or delaying the onset of sprouting in comparison with the controls, but the potato pieces used were non-dormant, and Hemberg suggested that these were capable of destroying the inhibitor.

The present work, begun before the papers of Burton (1956) and Varga and Ferenczy (1957 a) were available, was designed chiefly to investigate two main points, (1) the effect of the inhibitor on potato buds themselves, and (2) the correlation between stage of dormancy and amount of inhibitor as determined by a coleoptile straight growth bioassay.

Methods

Sampling of tubers. Red Pontiac, Plymouth, Katahdin, and Merrimack varieties were used, the first two having a fairly short dormant period, the third an intermediate one, and the last a long dormant period. Four storage temperatures, 32°, 40°, 50°, and 75°F, were used. Samples were taken immediately after harvest and approximately monthly thereafter until sprouts were visible. Each sample consisted of fifteen tubers.

The eyes from the fifteen tubers were removed with a scalpel. This included the buds and about 2 mm of tissue surrounding them. The eye tissue thus included some peel and inner portion of the tuber. The remainder of each tuber was halved lengthwise, from basal to apical end, and the peel, 1 mm thick, removed from one half of each tuber. This peel sample, therefore, contained no eye tissue. The peeled portion of the tubers were again halved lengthwise, and one half of each half-tuber (one fourth of the original tuber) diced into cubes one-half inch or smaller.

As each part of the tuber was removed, it was dropped into crushed dry ice and frozen. Samples were freeze-dried without being heated, then ground to 40 mesh in a micro Wiley mill. The ground powder was stored over a desiccant (silica gel) in the dark at 0°F until used.

At the same time that the fifteen-tuber composite sample was taken, ten more tubers were removed and placed in a chamber at room temperature and 75—85 % relative humidity. These tubers were examined periodically and the date on which one half (five tubers) had visibly sprouted was recorded.

Extraction and chromatography. The dry tissue was extracted with ether by the method of Wildman and Muir (1949), and separated into neutral and acidic fractions by the method of Bonde (1954). One-tenth or two-tenths gram dry eye tissue, or one-half gram of the peel or inner portion tissue, was used for extraction. The extracts were chromatographed with isopropanol, ammonium hydroxide, water (8 : 1 : 1) on strips of Whatman No. 1 paper two cm wide. After development, each strip was cut transversely into twenty equal segments, approximately one cm long. These chromatogram segments were assayed by either the *Avena coleoptile* straight growth test or by the potato eye plug test.

Avena coleoptile straight growth test. The general method of Nitsch and Nitsch (1956) was used with very little change. Vicar variety oats was substituted for Brighton. The oats were soaked for two hours in water and grown on paper tissues for sixty-eight hours, red light being given for six hours on the first day and for three hours on the second day. Twenty-five mm long coleoptiles were selected, the three mm at the tip discarded, and the next 4 mm section used for the bioassay. Coleoptile sections were soaked two hours in 1 mg/l MnSO_4 before being placed in test tubes containing one ml buffered 2 percent sucrose and a segment of the chromatogram. The tubes were rotated for twenty-four hours, the coleoptile sections removed, and their final length measured. Measurement was made with the aid of a double lens seven power magnifier containing a scale graduated in 0.1 mm.

Potato eye plug assay. A cylinder was cut through the center of the potato eye with a No. 3 cork borer. The most basal and most apical eyes on the tuber were omitted. The cylinder was removed and the surface end cut off about three mm from the center of the eye. The eye plug thus was a small cylinder seven mm in diameter and three mm in height, weighing about one-tenth gram, and containing several buds or growing points. The plugs were washed in running tap water for five hours before planting.

Polyethylene ice cube trays with individual ice cube compartments one cm square or smaller were used to grow the plugs. The paper chromatogram segments were each placed in the bottom of a compartment, and covered with 1.4 grams of fine quartz sand which had been sterilized previously. The plugs were then treated with Chlorox (1 : 3) for one minute, rinsed three times with sterile water, and placed in the sand with top surface exposed, two plugs to a compartment. Eight-tenths ml of the same buffer (pH 5.0) as used for the oat assay was added, and the trays stored in a chamber maintained at 58°—60°F. Water was added each day or when needed. The number of days until a sprout was first observed was recorded for each plug.

Size of tuber and position of the eye on the tuber did not appear to affect the length of time until visible sprouting of the plug.

Table 1. *Analysis of variance of days required for visible sprouting.* Potato plugs grown with blank papers and with chromatogram segments containing inhibitor from acidic ether extracts of Merrimack potatoes at three sampling dates.

Source of variation	Degrees of freedom	Sum of squares	Mean square	F	F required for significance	
					P = .05	P = .01
Total	59	1948.58	—	—	—	—
Replications	1	1804.41	1804.41	553.5	18.51	98.49
Samples	2	6.53	3.26	1.06	—	—
Replications \times Samples (Error A) ...	2	6.14	3.07	—	—	—
Segments	4	3.83	0.96	< 1	—	—
Samples \times Segments	8	23.97	3.00	1.21	2.17	2.96
Error B	42	103.70	2.47	—	—	—

Experimental Procedure and Discussion

Avena straight growth assay. Samples of eye tissue from potatoes at various stages of dormancy were assayed. Coleoptiles grown in solution in contact with those segments of the chromatogram from R_t 0.65—0.90 did not elongate as much as did similar coleoptiles grown in contact with blank segments of the chromatogram, thus indicating the presence of Hemberg's acidic growth inhibitor. Samples at various stages of dormancy did not differ significantly in the amount of inhibition present. Even in samples from tubers which had just begun to visibly sprout, an inhibition zone could be detected between R_t 0.65 and 0.90. However, variation among replicate samples was rather large, particularly in the chromatographic step because of variable R_t values, so that small differences in amount of inhibitor among samples could not be detected.

Hemberg (1949) reported that there was no greater concentration of acidic inhibitor around the eyes than in the rest of the peel, but in the potatoes used in this investigation there was approximately one-fifth as much acidic inhibitor in either peel or middle portion as in the eye tissue. The inhibitor was present in the peel and in the inner portion when the tubers had begun to sprout as well as immediately after harvest.

Potato eye plug assay. Segments of the chromatogram were also assayed with potato eye plugs. There was no effect on time of sprouting of the plugs with any segment of the chromatogram, including those segments which suppressed or promoted elongation of *Avena* coleoptiles. Table 1 shows analysis of variance of the results of the bioassay of chromatographed acidic extracts of eye tissue of Merrimack tubers sampled immediately after harvest, midway through the dormant period, and when sprouts first became visible. The segments analyzed were numbers 15—18 inclusive (R_t 0.70—0.90).

It is variation due to segments or to segments \times sample interaction which

Table 2. *Analysis of variance of days required for sprouting.* Potato plugs grown with blank papers and with segment no. 17 (R_t 0.80—0.85) of acidic ether extracts of eye tissue of dormant Pontiac tubers.

Source of variation	Degrees of freedom	Sum of squares	Mean square	F	F required for significance $P = 0.05$
Total	15	25.75	—	—	—
Replications	3	12.25	4.08	3.39	3.59
Segment 17 vs. Blank Segment	1	0.25	0.25	< 1	—
Error	11	13.25	1.20	—	—

would indicate whether the inhibitor delays sprouting of the plugs. Neither is significant. Replications are significantly different because the potato plugs used for the two replications were different. Plugs used in one replication began to sprout two days after being planted and in the other replication twelve days after being planted.

The chromatographed neutral ether extracts of potato eyes, also, failed to suppress or to promote sprouting of the plugs.

Since chromatogram segments from neither acidic nor neutral fractions caused a delay in sprouting of the buds on potato plugs, it was decided to see if unfractionated, unchromatographed ether extracts had any effect. Dry, powdered eye tissue was extracted with ether as usual, and the filtered extract applied directly to a piece of filter paper the same size as a chromatogram segment. This paper was placed at the bottom of a compartment of the plastic tray, covered with sand, and two eye plugs planted as usual. Similar pieces of paper were used for the blanks.

The number of days until a sprout was visible did not differ regardless of whether the plugs were in contact with blank pieces of paper or with unchromatographed extracts of eye, peel, or inner portion tissue. No delay in sprouting of the plugs could be detected even when they were grown in contact with extracts of freshly harvested tubers.

One-tenth gram of dried eye tissue contained at least 12.5 times as much actual bud tissue as did the two eye plugs themselves. Therefore, at least 12.5 times as much inhibitor was applied to the plugs as was present in the bud portion of the plugs themselves.

Re-extraction of added inhibitor from potato plugs. Since no delay in sprouting due to treatment with ether extracts of dormant tubers was detected, the question arose as to whether the inhibitor had actually entered the potato plug. It was possible that the plugs either did not take up the inhibitor, or that an enzyme was present which destroyed it. It is this latter possibility which Hemberg (1949) suggested was the cause of his negative results in the experiment in which he attempted to delay sprouting of pieces of eye tissue.

Table 3. *Analysis of variance of Avena bioassay.* Ether extracts of potato plugs grown on blank papers and on segment 17 (R_f 0.80—0.85) of chromatographed ether extract of dormant Pontiac potatoes. Analyzed as logarithm of length of coleoptiles.

Source of variation	Degrees of freedom	Sum of squares	Mean square	F	F required for significance	
					P = 0.05	P = 0.01
Total	47	0.057358	—	—	—	—
Replications	2	0.005817	0.002908	2.79	3.21	5.12
Segment 17 vs. Blank Segment	1	0.005749	0.005749	5.52	4.06	7.24
Error	44	0.045792	0.001041	—	—	—

In order to determine whether or not the added inhibitor was present in the eye plugs, the following experiment was conducted.

A fresh sample of freeze-dried eye tissue was prepared from Pontiac tubers which had just been harvested. The time until one half the tubers sprouted when transferred to 75—85°F was 32 days. *Avena* bioassay of the acidic fraction of ether extracts showed that as usual there was suppression of coleoptile growth due to the area of the chromatogram between R_f 0.65 and 0.90.

Potato plugs were grown on the chromatographed acidic fraction as they had been previously. There were four replications with two plugs for each segment of each replication. The potato plugs used for each replication were different, but all were near the end of the dormant period. No delay in time of sprouting was observed for plugs on any of the chromatogram segments. Table 2 shows the results of analysis of variance of the time until the plugs grown on segment number 17 (R_f 0.80—0.85) began to sprout.

As soon as each plug sprouted it was removed from the tray, rinsed free of sand, dropped into powdered dry ice, and frozen. When all the plugs had been frozen, they were freeze-dried and again extracted. Each pair of plugs was placed in a test tube with two ml ether and allowed to stand overnight at 32°F. Next day the ether was decanted, the plugs rinsed with fresh ether, and the combined ether extract evaporated at room temperature. Buffered sucrose was added to the dried extract and the *Avena* straight growth test conducted as usual. Plugs grown on the blank segments of the chromatogram were extracted in the same way. Table 3 gives analysis of variance, for three of the replications, of the length of coleoptiles grown with the extracts of plugs grown with blank segments and with the extracts of plugs grown with segment 17 (R_f 0.80—0.85). The data were transformed to logarithms to correct for non-homogeneity of variance.

Thus, even though the plugs showed no delay in time of sprouting, the

Table 4. *Analysis of variance of Avena bioassay.* Extracts of potato plugs which were grown with segments of chromatograms containing inhibitor and on blank segments. Chromatograms were prepared from acidic ether extracts of eye tissue of Merrimack tubers at different stages of dormancy. Analyzed as logarithm of length of coleoptiles.

Source of variation	Degrees of freedom	Sum of Squares	Mean square	F	F required for significance	
					P = 0.05	P = 0.01
Total	55	0.077592	—	—	—	—
Extracts	6	0.029047	0.004841	4.89	2.29	3.20
Error	49	0.048545	0.000991	—	—	—

Means: Sample A, segment 17=0.7854, segment 18=0.7846

Sample B, segment 17=0.8156

Sample C., segment 16=0.7516

Blank segments=0.8257, 0.8072, 0.8012

hsd=0.0492

acidic inhibitor was present in the plugs in large enough amount to be easily detectable when re-extracted and assayed by the *Avena* straight growth test. Since the inhibitor was present, but dormancy was not prolonged, the inhibitor does not appear to be the cause of potato dormancy.

The re-extraction procedure was repeated with plugs grown on one replication of the chromatographed extracts of the three samples of Merrimack tubers of Table 1. Sample A was taken at harvest, Sample B three weeks after harvest and Sample C eight weeks after harvest. The plugs were frozen, dried, and extracted with ether. *Avena* coleoptiles were treated with each extract. Analysis of variance of length of coleoptiles grown with the segments causing greatest suppression of growth of the coleoptiles is shown in Table 4.

Using Tukey's hsd=0.0492, there is no difference in length among the coleoptiles treated with extracts of the three blank-grown pairs of plugs. Coleoptiles treated with extract from the sample C segment differ from those treated with extracts from the blanks. There is no difference between coleoptiles treated with extracts from the two individual segments of sample A, but if one assumes that the inhibitor is equally distributed between the two segments, and adds the amount of suppression of growth, the sum of the two is 0.0528 which exceeds the hsd=0.0492. Length of coleoptiles treated with extract from the sample B segment does not differ from the length of those treated with extracts of plugs grown on blanks. There are several possible reasons for failure to detect the inhibitor upon re-extraction of sample B. Extractions of the plug may have been incomplete. Mold or rot

may have been present on the plugs but not detected, and these organisms may have destroyed the inhibitor. Since the inhibitor was recovered in extracts of plugs treated with the other two samples, and in extracts of three sets of plugs treated with the Pontiac sample, it seems unlikely that the inhibitor from sample B was destroyed by enzymatic action of the potato tissue.

Added indoleacetic acid (IAA) could also be recovered from potato plugs, but not quantitatively. Pieces of filter paper containing 0, and 0.1, and two pieces each containing 1.0 microgram of IAA were placed at the bottom of ice cube tray compartments and plugs grown as usual. No effect on time of sprouting was detected. The plugs were removed, frozen, dried, re-extracted, and assayed by the straight growth test. The mean length of coleoptiles was 6.48, 6.62, 6.70 and 6.85 mm for 0, 0.1 and the two 1.0 microgram concentrations, respectively. Although obviously not quantitative recovery, the mean of the two 1.0 micrograms treatments was significantly higher than the treatment containing no IAA. Some of the added IAA apparently was present in the potato plugs, and when extracted, affected the growth of oat coleoptiles. For a substance as easily destroyed as IAA is reported to be, quantitative recovery could not be expected.

Acidic ether extracts of potato eyes were found to contain a substance which inhibits elongation of *Avena* coleoptiles. This substance did not disappear when the potato began to sprout, but even if it had, that fact would not, of itself, be proof that the inhibitor causes potato dormancy. As pointed out by Burton (1956), the concentration of many compounds in potatoes is roughly correlated with stage in the dormant period. The only real test of the action of an inhibitor in causing dormancy is its effect in delaying the time required for potato eyes to sprout. The acidic inhibitor referred to by Hemberg causes no such delay in sprouting, even though it can be shown to be present in the eye tissue. Potato dormancy therefore, does not appear to be due to the presence of this inhibitor.

If the inhibitor is a phenolic or fatty acid as suggested by Varga and Ferenczy (1957 b) or Bentley (1958), its effect on coleoptile elongation may be a matter of concentration rather than of specific inhibitory action. Two to ten coleoptiles, each four mm long, were treated with the inhibitor present in one-half gram (fresh weight) of potato tuber eye tissue. For many substances this probably is a much higher concentration than would ordinarily be found in an oat or wheat coleoptile. The inhibitory effect may be due simply to too high a level of some substance which might even stimulate growth at levels at which it normally occurs in coleoptiles.

Summary

Hemberg (1947) reported that there was an acidic growth inhibitor present in the peel of freshly harvested potatoes, which disappeared when the potatoes were stored for several months. He suggested that the presence of the inhibitor is responsible for the failure of potatoes to sprout immediately after harvest. The present work was undertaken in order to investigate further this theory of Hemberg.

The amount of acidic inhibitor in the area of the peel around the eyes of potato tubers at various stages in the dormant period was investigated using the *Avena* coleoptile straight growth test. Four varieties of potatoes were stored at four temperatures and sampled at approximately monthly intervals from time of harvest until sprouted. The buds plus the tissue immediately surrounding them were extracted with ether. The extracts were separated into acidic and neutral fractions and chromatographed on paper with isopropanol, ammonium hydroxide, water (8 : 1 : 1). Segments of the chromatograms were assayed by the *Avena* coleoptile straight growth test. No difference in content of acidic inhibitor was found among potatoes in various stages of dormancy.

Sprouting of potato buds was also used as a bioassay. Pieces of potato tissue weighing about one-tenth gram and containing an eye were planted in sand in aqueous solution in contact with segments of the chromatograms. The time until a sprout was visible was recorded for each eye piece. No delay in sprouting was found when eye pieces were treated with chromatogram segments containing the inhibitor. Treatment with the unchromatographed ether extract also failed to cause a delay in sprouting of the eye pieces.

Peels from which the eyes had been removed and the inner tissue of the tuber each had approximately one-fifth as much acidic growth inhibitor by *Avena* assay as did the eye, or bud tissue. No delay in sprouting of eye pieces was found upon treatment with ether extracts of peel and inner tissue of tuber.

Eye pieces treated with chromatogram segments containing the acidic inhibitor and pieces treated with blank segments of the chromatogram were extracted with ether and the extract assayed by the *Avena* coleoptile straight growth test. Extracts of those pieces which had been in contact with chromatogram segments containing the inhibitor caused a suppression of coleoptile growth. The applied inhibitor thus was present in the eye pieces, although no delay in sprouting had occurred. It was concluded that the presence of the acidic growth inhibitor is not the cause of failure of freshly harvested potatoes to sprout.

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Indole-3-acetic Acid as Protective Substance against X-rays

By

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Modification of the effects of ionizing radiations by various substances constitutes a large field of present-day investigation. Special interest attaches to the substances which are found naturally in organisms, since experiments with them might throw light on the causes of the great variation shown by the organisms in their reaction to radiations.

In connection with other x-ray experiments it became evident that indole-3-acetic acid (IAA) offers some protection against the effects of x-rays. In the following report a series of experiments showing this effect are described.

Materials and Methods

As plant material root tips of *Narcissus tazetta* L. "Totus albus" were used. The bulbs were placed over 350 ml jars filled with tap water. When the roots had reached a length of 5—10 cm they were used for the experiments.

The irradiation was carried out at the Central Institute of Radiotherapy, Radio-physical Laboratory, Helsinki. For generous help the authors are greatly indebted to Mr. K. Salimäki, M. Sci.

The dose in all the experiments was 200 r. The potential of the current was 190 kv and it was screened through a 1.0 mm Cu filter and applied at a distance of 40 cm from the tube.

The treatments with IAA were carried out as follows. The roots grown in tap water were transferred to the solution to be tried 4 hrs prior to the irradiation and also irradiated in this solution. Immediately after the irradiation the roots were placed in tap water to recover. The IAA solutions were prepared by dissolving the

Table 1. *The effect of 200 r x-rays on the root tip cells of Narcissus tazetta.* In experiment 1 only x-rays have been used; in the others prior to the irradiation a 4-hr treatment with the indicated concentration of IAA was given.

Experiment No.	Concentration of IAA (ppm)	No. of normal anaphases	No. of anaphases with aberrations	Percentage of anaphases with aberrations	No. of bridges	No. of fragments	No. of minute fragments	No. of laggards
1	—	103	74	41.2	40	49	61	17
2	10	82	48	36.9	25	38	22	20
3	20	129	80	38.3	38	54	49	16
4	30	61	31	33.7	14	15	15	17
5	50	64	31	32.6	20	21	21	9
6	70	270	88	24.6	71	63	45	18

substance in a few drops of absolute ethanol which was diluted with distilled water to the volume wanted. The concentrations used were 10, 20, 30, 50, and 70 ppm.

The treatments and the irradiations were carried out at room temperature, *i.e.* 19–21°C.

As fixative acetic-alcohol (1:3) was used, and the root tips were squashed according to the usual Feulgen technique (Darlington and La Cour 1947).

Root tips were fixed 1, 2, 3, and 4 days after irradiation. Similar preparations were made also of untreated root tips and of root tips after the growth substance treatment but before the irradiation.

Results

The chromosome number of *Narcissus tazetta* used in the present experiments was $2n=22$. This number is too high to permit an analysis of chromosome changes in metaphase. Anaphase stages were therefore used to analyze the effects of the treatments. The following aberrations were scored separately: bridges, fragments, minute fragments and laggards. No more complicated configurations than these were observed in the anaphases. It is obvious that a proportion of the bridges and fragments belong together, owing their origin to the same sequence of chromosome changes. This is suggested by the fact that the number of bridges agrees with the number of large fragments (Table 1). This, however, could not be taken into consideration when the aberrations were scored.

In the untreated root tips no abnormal mitoses were observed. After 4 hrs treatment with any of the IAA concentrations used mitoses were about as frequent as in untreated roots. These, too, were quite regular.

The results of the scoring (Table 1) show that the percentage of aberrant anaphases diminishes with increasing IAA concentration. The statistical

significance of the results has been determined by plotting each figure against all the others. This calculation shows that the difference in the results between the irradiation without IAA treatment (experiment 1) and the treatment with 70 ppm IAA (experiment 6), between the treatment with 10 ppm IAA (experiment 2) and with 70 ppm IAA (experiment 6), and between the treatment with 30 ppm IAA (experiment 3) and with 70 ppm IAA (experiment 6) are significant ($P < 0.01$). Since all the results point in the same direction, it seems safe to conclude that the IAA treatment has a protective effect against x-rays. This effect is, however, not strong.

Discussion

A great proportion of the substances which show a protective action against ionizing radiation have been found to be reducing agents (Dale 1954, Guzman Barron 1954, Riley 1954, Bacq and Alexander 1955). Their protective activity has been interpreted in terms of their ability to react with oxygen or the oxidizing radicals which arise when water is ionized by radiations. That other mechanisms may play a role is shown by the fact that experiments *in vitro* and *in vivo* often give different results. In the words of Bond and Cronkite (1957, p. 302): "It cannot be said at this time, in complex systems, whether the agent injected has changed the biological substrate to a more radioresistant state, whether actual competition for free radicals is underway, or whether production of peroxides, etc., has been diminished by an oxygen deficit."

The present experiments show that IAA has a slight protective effect against chromosome breakage with x-rays in the root tip cells of *Narcissus tazetta*. It is known that the mitotic stage at which the cell is irradiated affects the radiation damage (Timonen 1953). In the present experiment, however, the IAA had not acted long enough to change the ratios of the different mitotic stages, and its protective action cannot therefore be interpreted in these terms.

Since the experiments of Skoog (1935) concerning the effects of x-rays on plant growth and auxin destruction, we know that x-rays destroy IAA both *in vivo* and *in vitro* and that the inhibition of plant growth caused by the radiation can be counteracted by added auxin. Since auxin is known to be an oxidizable substance (Ray 1958), its destruction by radiation has been assumed to be an oxidation reaction.

The protective effect shown by IAA might accordingly be interpreted as depending on its reaction with oxidizing radicals and hydrogen peroxide caused by radiations. However, an alternative explanation might also be

considered. The presence of auxin appears to be a prerequisite both for deoxyribonucleic acid synthesis in plant nuclei and for their mitotic division (Das, Patau and Skoog 1956, 1958, Patau, Das and Skoog 1957). It might therefore be possible that IAA in some — as yet unknown — way affects the chromosome breakage caused by x-rays by modifying the nucleic acid metabolism of the nuclei (Gordon 1957).

Summary

IAA in concentrations of 10, 20, 30, 50, and 70 ppm has been found to have an increasingly protective effect against x-rays (200 r) in the root tip cells of *Narcissus tazetta* L. Two possible explanations for this effect are suggested: As an oxidizable substance IAA may react with oxidizing radicals and hydrogen peroxide caused by the radiation, or IAA may affect chromosome breakage by influencing the deoxyribonucleic acid metabolism of the nuclei.

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Action de la longueur du jour sur la croissance d'*Anastatica hierochuntica* L.

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Introduction

En 1957 (Binet 1957), la culture à Paris de Rose de Jéricho (*Anastatica hierochuntica* L.), sur solution nutritive, nous a permis d'interpréter la morphologie de cette espèce saharienne et de montrer combien sa croissance est sensible aux variations de l'intensité lumineuse. Nous avons poursuivi nos recherches sur le comportement physiologique de cette annuelle désertique, en étudiant le développement d'individus soumis à des photopériodes différentes. Les résultats exposés ci-dessous sont relatifs à la croissance de l'appareil végétatif.

Matériel et techniques

Les graines utilisées ont été extraites de fruits récoltés près de Béni-Abbès (Sua Oranais), sur des pieds desséchés d'*Anastatica hierochuntica*. Ces semences sont toutes germées après 24 heures de séjour sur papier filtre humide, à 22° C, à la lumière du jour. Au bout de 10 jours, les jeunes plantes dont seuls les cotylédons sont épanouis, sont repiquées en pots, dans du sable de dune. Elles sont alors réparties en 4 lots. Chaque lot comprend 60 plantes. Tous les lots sont placés dans les mêmes conditions de température (20—25°), d'humidité atmosphérique (60—70 % d'humidité relative) et d'arrosage. Ils sont tous éclairés artificiellement par des unités Phytorel (4 tubes Phytor III) de telle sorte qu'ils reçoivent un éclairément de 4.000 lux. Seule la durée de l'éclairément varie d'un lot à l'autre, les photopériodes utilisées étant de 8, 12, 16 et 24 heures.

Nous avons déjà décrit les modalités générales de la morphogénèse chez *Anastatica hierochuntica*.
Physiol. Plant., 12, 1959

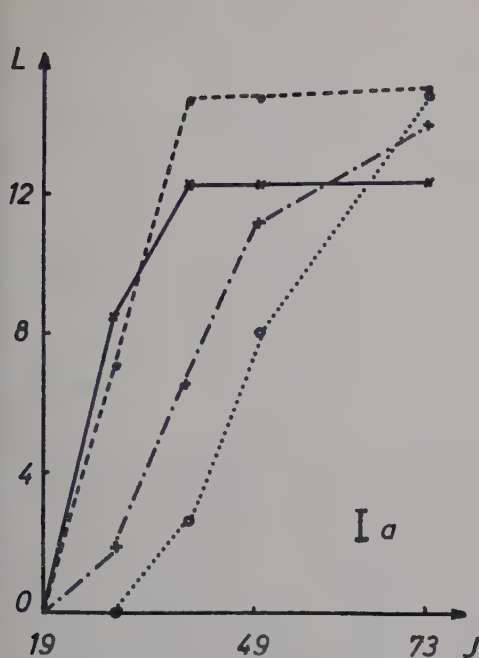


Figure 1.

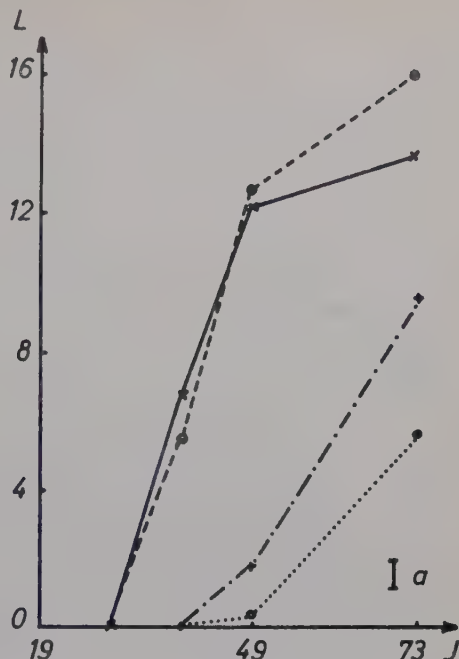


Figure 2.

Figure 1. Variations de la longueur (L , en mm) de l'entre noeud T1 en fonction de l'âge (J , en jours) des plantes développées en photopériode de 24 (\times), 16 (\bullet), 12 ($+$) et 8 heures (\circ) (a =amplitude de variabilité).

Figure 2. Variations de la longueur de l'entre noeud T3 en fonction de l'âge des plantes (mêmes notations que pour la figure 1).

tica (Binet 1957). Rappelons simplement que l'épicotyle se développe en une tige principale portant quelques feuilles (2 à 6 en général). A l'extrémité de cette tige apparaît une inflorescence. Dès lors, ce sont les bourgeons situés à l'aisselle des deux feuilles les plus jeunes qui s'allongent en rameaux feuillés, donnant à la plante un aspect bifurqué. La formation de cette bifurcation est donc lié à l'apparition des premières fleurs.

Chez les plantes jeunes, on a étudié la croissance des 4 premières feuilles (F1, F2, F3, F4) dont on mesure la longueur totale (limbe + pétiole) tous les 10 ou 20 jours, et l'allongement des entre-noeuds T1 et T3 respectivement compris entre les cotylédons et F1, et entre F2 et F3; l'entre noeud T2, entre F1 et F2, restant toujours très court (3—4 mm au maximum) n'a pas été considéré.

Pour les plantes âgées de plus de 45 jours, la croissance a été suivie en étudiant l'augmentation du poids frais et du poids sec d'une plante entière.

Tous les résultats exposés ci-dessous représentent la moyenne d'au moins 20 mesures. Sur les graphiques l'amplitude des variations a été notée sous forme d'un petit segment parallèle à l'axe des ordonnées. Les âges (exprimés en jours) sont comptés à partir du repiquage et du passage des plantes en photopériode.

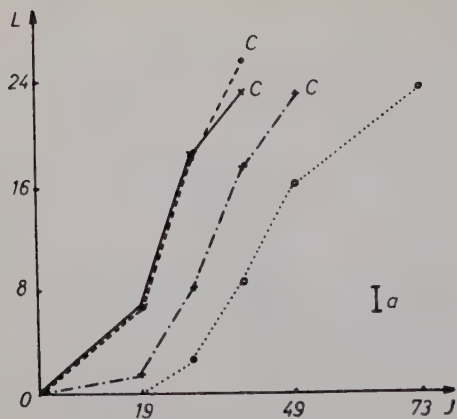


Figure 3.

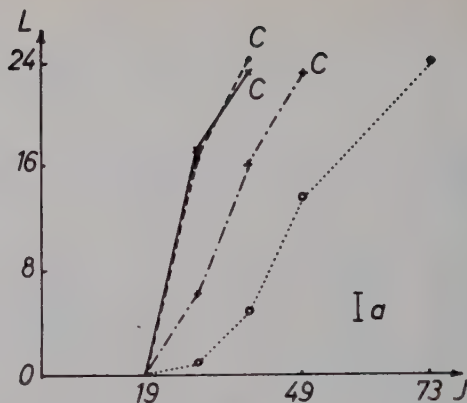


Figure 4.

Figure 3. Variations de la longueur de la feuille F1 en fonction de l'âge des plantes (mêmes notations que pour la figure 1). C=chute des feuilles.

Figure 4. Variations de la longueur de la feuille F2 en fonction de l'âge des plantes (mêmes notations que pour la figure 3).

Résultats

1. *Croissance des entre noeuds.* Les figures 1 et 2 représentent les variations de la longueur des entre noeuds T1 et T3 en fonction de l'âge des plantes. On constate que la vitesse de croissance de ces portions d'axes est sensiblement la même en 24 et en 16 heures. Après le 29^{me} jour, pour cette dernière photopériode, l'allongement s'accélère quelque peu et mène à la construction d'entre-noeuds un peu plus longs que ceux observés en lumière continue. Par contre en 12 et 8 heures, l'allongement de ces entre-noeuds commence tardivement et se réalise lentement. En 24 et 16 heures, T1 a atteint sa longueur définitive au 39^{me} jour. En 12 et 8 heures, ceci n'est réalisé qu'au 73^{me} jour. Dans ces conditions, T3 ne commence à s'allonger que vers le 49^{me} jour alors que sous l'action des journées longues, la croissance de cet entre noeud est déjà pratiquement terminée.

2. *Croissance des feuilles.* L'observation des figures 3, 4, 5 et 6 montre que la croissance des feuilles est la même en 24 et 16 heures. En jours courts, elle est d'autant plus tardive et plus lente que la durée du jour est plus petite. On peut remarquer également qu'en 24 et 16 heures les feuilles F3 et F4 se développent ensemble et à la même vitesse alors qu'en 12 et 8 heures, F3 précède quelque peu F4 dans son allongement. De plus, en journées longues, les 4 premières feuilles tombent rapidement. En 12 heures, seules les feuilles F1 et F2 sont précocement caduques. En 8 heures, aucune de ces feuilles n'est tombée au 73^{me} jour.

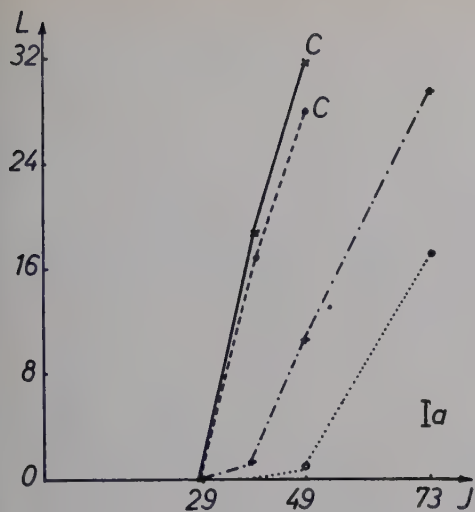


Figure 5.

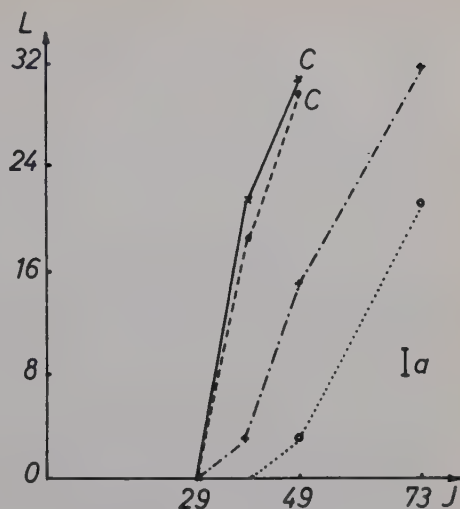


Figure 6.

Figure 5. Variations de la longueur de la feuille F3 en fonction de l'âge des plantes (mêmes notations que pour la figure 3).

Figure 6. Variations de la longueur de la feuille F4 en fonction de l'âge des plantes (mêmes notations que pour la figure 3).

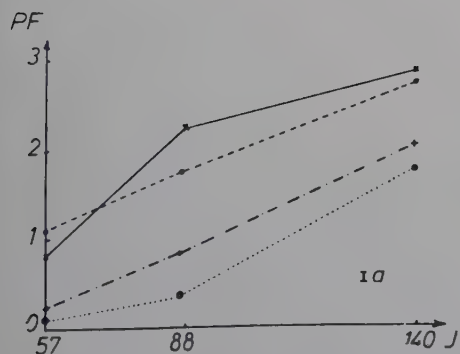


Figure 7.

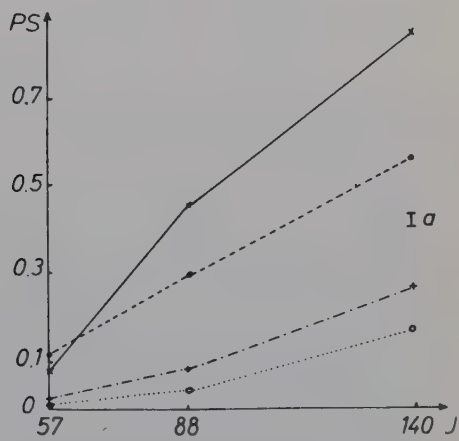


Figure 8.

Figure 7. Variations du poids frais (PF, en g) d'une plante en fonction de son âge (mêmes notations que pour la figure 1).

Figure 8. Variations du poids sec (PS, en g) d'une plante en fonction de son âge (mêmes notations que pour la figure 1).

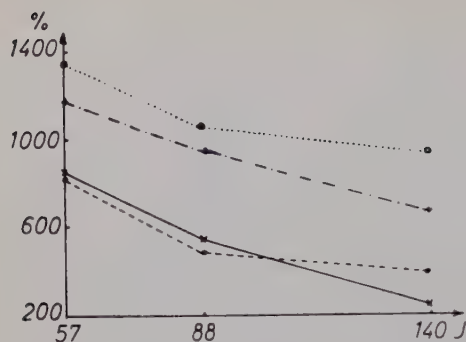


Figure 9. Variations du rapport 100 eau/matière sèche d'une plante (% en ordonnées) en fonction de son âge (mêmes notations que pour la figure 1).

3. *Poids frais et poids sec des plantes entières.* Entre le 57^{me} et le 140^{me} jour, le poids frais et le poids sec des plantes augmentent régulièrement (voir les figures 7 et 8). Si à ce point de vue, au 57^{me} jour, ce sont les individus développés en 16 heures qui sont les plus importants, peu après, on constate que l'accroissement en poids est d'autant plus accusé que le jour est plus long. On peut remarquer que les différences relatives entre les plantes des divers lots sont beaucoup plus importantes si on considère les quantités de matière sèche plutôt que le poids frais. Ceci est dû au fait que la teneur en eau de la matière végétale n'évolue pas de la même façon chez les individus développés sous les différentes photopériodes utilisées (voir la figure 9).

Interprétations et discussions

Des faits précédemment exposés, nous pouvons déduire que chez *Anastatica hierochuntica*, la croissance est fortement ralentie lorsque la longueur du jour est de 12 ou 8 heures. Par contre pour les plantes jeunes, le développement est aussi bon en 24 qu'en 16 heures. Mais après avoir atteint un certain âge, les plantes croissent le plus activement en lumière continue. Pour cette espèce il semble donc exister, comme nous l'avons déjà mis en évidence chez une autre plante saharienne, *Zilla macroptera*, (Binet 1955), deux stades successifs pendant lesquels les réactions des végétaux vis à vis du milieu extérieur peuvent être différentes. De ce fait, nous sommes amenés à revenir sur l'interprétation que nous avons donnée, de nos expériences de 1957. Nous avons en effet constaté en cultivant, sous un abri vitré, non chauffé, des *Anastatica*, les uns au soleil, les autres à l'ombre, que les différences observées entre les 2 lots d'individus s'accroissaient fortement à partir du mois de mai (plantes alors âgées de 60 jours). Nous avons alors pensé qu'avant cette date, c'étaient les températures relativement basses qui

avaient limité la croissance et empêché l'action de l'intensité lumineuse. En fait, dans les expériences exposées ci-dessus, pendant lesquelles la température n'a pas varié, nous constatons que les plantes âgées de 60 jours environ, modifient leurs réactions vis à vis des facteurs externes. La température ne pouvant être mise en cause, seule une modification dans les propriétés de la matière végétale semble devoir être retenue.

La longueur des jours règle la vitesse de la croissance des entre-nœuds et des feuilles. Mais elle ne modifie pas la longueur finale de ces organes. En particulier la taille et la forme des feuilles sont les mêmes quelle que soit la photopériode appliquée. On ne retrouve donc pas là d'action de la longueur du jour sur la morphogénèse des feuilles comme c'est le cas avec de nombreuses autres espèces: *Kalanchoe Blossfeldiana* (Harder et Witsch 1940), *Sedum Kamtchaticum* (Meyer 1947), *Sesamum orientale* (Sen Gupta et Payne 1947), *Sinapis alba* (Bronchart 1957), *Ulex europaeus* (Binet 1958) par exemple. Ce fait est à rapprocher de nos observations précédentes (Binet 1957) faites sur des *Anastatica* développés à des intensités lumineuses différentes et chez lesquelles les feuilles ont toutes la même forme et la même surface. Le milieu extérieur modifie donc surtout la vitesse d'apparition et d'épanouissement des feuilles sans toucher à l'aspect final de ces organes.

Au Sahara, les Roses de Jéricho se développent au printemps, au moment où les jours sont les plus longs (14 heures environ). Il est donc vraisemblable que la photopériode est un des facteurs climatiques qui permet le rapide et important développement des individus. Remarquons cependant que nos expériences ont eu lieu sous un éclairage de beaucoup inférieur à celui qui règne au Sahara. Au désert, l'intensité lumineuse a une action certainement beaucoup plus importante que celle de la longueur du jour.

Sommaire

Des *Anastatica hierochuntica* ont été cultivées à la même température, dans les mêmes conditions d'humidité du sol et de l'air, sous un même éclairage, mais à des photopériodes de 8, 12, 16 ou 24 heures.

La croissance des plantes jeunes est caractérisée par l'allongement des entre-nœuds basaux et des 4 premières feuilles. Chez les plantes âgées, on suit l'augmentation du poids frais et du poids sec.

La longueur du jour n'a pas d'action sur la longueur finale des entre-nœuds, la forme et la taille définitives des feuilles. Par contre la croissance est fortement ralentie en 8 et 12 heures. Chez les plantes jeunes, la croissance est aussi active en 16 heures qu'en lumière continue. Pour les plantes âgées d'au moins 60 jours, la croissance est maximum en lumière continue.

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Bio-Regulatory Activity and Nitrogen Function in Organic Compounds.

Antioxidant Properties and their Physiological Significance

By

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I. Introduction

1. *General considerations on oxygen and antioxidants.* — The recent investigations of Gerschman, *et al.* (11 to 17) and others (2, 7, 9, 24, 29) have revived interest in the old problem of oxygen-poisoning. Some studies have implicated free-radical intermediates as common both to oxygen and x-ray-initiated damage. The fact that it is often necessary to study oxygen poisoning at partial pressures above the atmospheric level is a matter of experimental technique that in no way reduces the significance of the concept of oxygen toxicity. Further, it has been suggested that oxygen effects comprise a continuum which extends into the range of atmospheric oxygen tensions, and below. In support of this concept are: the inhibition of animal cell growth at atmospheric oxygen tensions; the behavior of hemoprotein-deficient anaerobes such as *Clostridium* spp.; the existence of microphilic aerobes such as *Azotobacter* spp.; and the beneficial effects of submergence upon germination and early seedling growth of rice and other plant species (1).

It has also been suggested that the Pasteur Effect may be a manifestation of oxygen toxicity (15), and it has been proven that physiologically essential enzymes — many containing sulfhydryl groups — are inactivated by oxidizing agents and even by aeration alone (19).

Out of research both on oxygen poisoning and radiation injury has come an imposing list of chemical substances able to serve in the role of biological

protectants. Marked reduction in damage by both types of agents results when organisms are treated with thiols such as glutathione or cysteine; phenols such as propyl gallate or nor-dihydroguaiaretic acid; the cobaltous ion; and other compounds (10, 11, 12, 17, 29).

Recognition of the low effective concentrations of protectants prompts regarding them as antioxidants. In contrast are the less active reducing agents, which react with oxidants in small whole number molar ratios. The physiological term "antioxidant" has not in general been defined rigorously, as for example by demonstration of the ability of low concentrations of protectants to delay oxidation of model substrates. A consideration of the agencies against which protection is directed, and their chemical constitution would seem to justify the biological use of the term antioxidant. It is likely that biological protectants operate as chemical antioxidants, serving as reaction-chain breakers (free radical "traps") as is NO in gas-phase reactions (30), or as cyclical inhibitors, as in the Co^{2+} — Co^{3+} system: Co^{3+} is formed during reduction of one hydroperoxide molecule and in turn is reduced again to Co^{2+} as it oxidizes a second hydroperoxide molecule (32).

The commonly recognized antioxidants native to organisms include glutathione, coenzyme A and cysteamine; nordihydroguaiaretic acid and tannins; ascorbic acid; cobaltous ion; and the vitamins E (11, 12, 14).

In a general sense, the mode of protection by antioxidants may be known, but the molecular species actually protected cannot now be specified, although they may be readily surmised. The oxygen effect most far-reaching in its consequences involves genetic material; (a) deoxyribonucleic acid exposed to oxygen in the presence of an H_2O_2 -yielding H-source (*e.g.*, RSH) undergoes a marked fall in viscosity (17), a phenomenon also encountered upon exposure of DNA to x-radiation (4); (b) Oxygenation of actinomycete mycelial slurries resulted both in direct oxidative attack upon DNA and its depolymerization (6).

Disaggregation of other macromolecules by exposure to elevated oxygen tension is known. Thus Na-Alginate with O_2 and H-Donor, or with H_2O_2 alone undergoes a marked drop in viscosity (17). Attack by an oxidant upon proteins would involve oxidation of thiols and aromatic nuclei (tyrosyl, tryptophyl residues), altering major protein characteristics such as catalytic or immunochemical properties. Oxidation of structural and reserve lipids in addition to rendering them non-functional, would produce an additional harmful intracellular oxidant. Thus ethyl oleate peroxide oxidizes thiols to disulfides and sulfoxides (8).

It is not necessary to detail other molecular types and specific molecules susceptible to oxidation, but it may be noted that interruptions in H-Transport systems by oxidants could alter the balance of respiratory products,

and increase in cellular peroxide level would lead to increased enzymic peroxidation of a variety of molecules including indole-3-acetic acid (IAA). Alteration in organic acid metabolism of *Bacillus asterosporus* by the cobaltous ion has been reported, and reduction both in IAA oxidation and tissue damage in peas by cobaltous ion has been linked to its antiperoxigenic action (5, 10).

2. *Organic nitrogen compounds as antioxidants.* It was noted that IAA and mescaline, 3,4,5-trimethoxyphenylethylamine (27, 28), markedly influenced the rate of air-oxidation of pyrogallol. These active molecules considered together with many hormones and other biological agents having little in common save for organic N suggested to us the possibility that N-compounds or N-containing groups present might be implicated in the regulatory functions of these compounds in organisms.

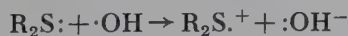
Support for this idea stemmed from the ability of amines to react with peroxides through transfer of an electron from the unshared pair of N (III) to the peroxidic bond or, alternatively, to a free OH radical. This reaction, the Horner-Schwenck mechanism of peroxide decomposition (32) may be exemplified by



Selection of a tertiary amine emphasizes electron-transfer rather than H-abstraction as the mode of antioxidant action.

To function with high stoichiometric efficiency, this reaction must remove chain-reaction initiators or propagators, or alternatively the product, $R_3N.^+$ must itself be able to regenerate the original amine by abstracting an electron from a second radical or other source.

Donation of an electron from an unshared pair is by no means a unique property of the N atom, but among the compounds of common biological occurrence those containing N and S appear able to react with especial facility in this manner. Thus



represents conversion of a thioether to an alkylsulfonium ion (25) concurrently with OH radical reduction.

3. *Chemical and biological evaluation of N-containing antioxidants.* The investigations to be recounted were based upon limited experimental observations, greatly fortified by general physiological, and especially chemical arguments as outlined above.

The objects of the present work are, therefore (a) demonstration of antioxidant properties for biologically active N-containing molecules and their relatives; (b) extension of this property over a reasonable wide range of

conditions; and (c) demonstration of physiological activity among compounds with chemically established antioxidant properties.

Thus, the chemical aspects are emphasized here, detailed physiological effects being reserved for future investigations.

II. Activity of N(III) Compounds in Chemical Systems

1. *Iodide oxidation.* Because of its relative simplicity the reaction $2\text{I}^- + \text{H}_2\text{O}_2 + 2\text{H}^+ \rightarrow \text{I}_2 + 2\text{H}_2\text{O}$ was selected for testing of antioxidants. Iodine formation was followed photometrically at 358 m μ (Bausch and Lomb Spectronic 20 spectrophotometer), an absorption peak of I_3^- , both in water and ethanol. Standard concentrations of 10^{-2} M KI and 10^{-3} M H_2O_2 were used.

In redistilled ethanol, iodide peroxidation was accelerated by catalysts, both $\text{Mo}_7\text{O}_{24}^{6-}$ and Fe^{3+} being suitable. The latter was selected in preference to the more complex molybdate-catalyzed system, and was used at 10^{-5} M.

The reaction in ethanol proceeded rapidly to completion at 25°C, approximately the theoretical amount of I_3^- for 10^{-3} M H_2O_2 being formed (Figure 1, control curve). Curves are based upon quadruplicate determinations (maximum standard error ± 1 % of the mean).

In the presence of 10^{-4} M IAA ($\text{H}_2\text{O}_2/\text{IAA}=10$) oxidation is markedly inhibited. Thus, the initial control rate of oxidation is 2.22 ± 0.1 Moles Liter $^{-1}$ min $^{-1}$ but in the presence of 10^{-4} M IAA M falls to about one-tenth the control value. Increase of IAA to 10^{-3} M lowers the initial rate somewhat more and greatly increases time required for attainment of the endpoint.

Interpretation of inhibition in the Fe catalyzed system is necessarily somewhat equivocal, as the number of possible interactions involving IAA must include those between Fe^{3+} and IAA. Hence, a modification of the I^- - H_2O_2 system was sought in which I_2 formation could be measured without catalyst or, preferably, in a catalyst-intensitive reaction mixture.

A suitable system was devised in aqueous medium with 10^{-2} M KI, 10^{-3} M H_2O_2 , and $M/15$ phosphate buffer, pH 6.65 (Figure 2). Neither Fe^{3+} nor molybdate at 10^{-5} to 10^{-4} M affected the reaction. With elimination of metal ion sensitivity, effects of IAA and other N-compounds could be interpreted independently of interactions involving metal ions.

Variability in this system was somewhat higher than that found for the ethanolic system (± 3 %, maximum standard error on the basis of over 50 duplicate determinations).

IAA (10^{-4} M) inhibited oxidation by about 20 % during the first two minutes of reaction (initial rate), the value increasing to somewhat more than 40 % after 6–10 minutes. Skatole (3-Methylindole), at 10^{-4} M is seen

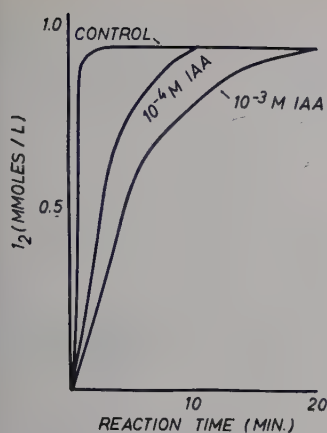


Figure 1.

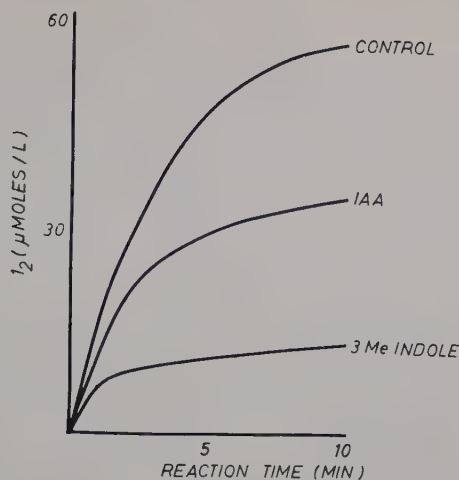


Figure 2.

Figure 1. Course of Fe-Catalyzed Iodide Peroxidation in Ethanol and its Inhibition by IAA.
 Figure 2. Course of Uncatalyzed Iodide Peroxidation in water at pH 6.65 and Inhibition by IAA and Skatole (3-Methylindole).

to be substantially more active than IAA, exhibiting an increase in activity with time.

On comparing indole and various indole derivatives, a kinetic distinction was observed, which divided these compounds into two general types. The first (Figure 2) is represented by inhibitors whose activity increases as the reaction proceeds. The second type, represented by 1- and 2-methylindoles and indole itself exhibits an interval of complete inhibition (lag period) followed by the beginning of I_2 formation (fig. 3). Observations made upon various indole compounds are therefore expressed in terms of (1) reduction in initial (2 min) rate at $10^{-4} M$ (H_2O_2 /inhibitor=10); (2) length of lag period, if present; and (3) rate relative to control in the first 2 min of reaction after termination of the lag period (Table 1).

Indole and its 1-, 2- and 4-methyl derivatives share high activity in the presence of a lag period. In contrast with substitution at all other positions tested, methylation of the 3 position reduces activity and alters kinetic behavior. If the 3-methyl group is now exchanged for an electron-withdrawing carbonyl group as in indole-3-aldehyde, all activity is abolished at $10^{-4} M$. A milder effect was obtained with the carboxyl carbonyl separated from the ring by one or more methylene groups, (IAA and IBA).

Activity (*i.e.*, effect on initial rate) was restored to the "deactivated" skatole (*i.e.*, IAA) by conferring phenolic character upon the benzene moiety, as in

Table 1. Comparative antioxidant activities of indole and substituted indoles.

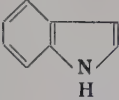
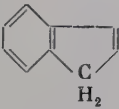
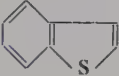

Substituted indole		Inhibition of oxidation at 10^{-4} M during 1 st 2 min (%)	Lag period (min)	Rate 1st 2 min after lag (% of control rate)
Group	Position			
None	(Indole) ₂	100	4	9
—CH ₃	1	100	10	4
	2	100	12	12
	3	65	0	—
	2, 3	64	0	—
	4	100	8	2
—CH ₂ CH ₂ NH ₂	3	96	0	—
—CH ₂ CH ₂ NH ₂	3	100	4	10
HO—	5		—	—
—CHO	3	0	—	—
—CH ₂ COOH	3	21	0	—
—CH ₂ COOH	3	100	4	7
HO—	5		—	—
—CH ₂ COOH	3	22	0	—
C ₆ H ₅ CH ₂ C—	5	25	0	—
—(CH ₂) ₃ COOH	3		0	—

5-hydroxy IAA which exhibits a lag period of short duration. Appropriately, removal of labile H from the foregoing, in 5-benzyloxy-IAA, restores quantitatively the activity of IAA itself. Loss in activity associated with the 3-alkyl group was largely offset by introduction of a terminal amino group, as in indole-3-ethylamine (tryptamine) and, as would be anticipated, further addition of phenolic character, in 5-hydroxy tryptamine (serotonin) restored full activity and the lag period.

Additional points of interest among indole compounds include: the relative effect of 2-substituent as seen in 2-methyl and 2-phenylindoles which at 10^{-5} M are more active than indole, and in oxindole (2-Keto-group) which is inactive. These points suggest that antioxidant activity is increased by electron-releasing agents (2-methylindole, possibly 2-phenylindole) and vitiated by electron-withdrawal in oxindole, in agreement with the arguments advanced for indole aldehyde.

Considering the limited group of compounds treated, a definite pattern of structure-activity relationships is strongly indicated. These data do not point to the significance of the N-atom as much as they emphasize the strategic character of the 3-position. With the gross form of the indole nucleus retained it is also of interest to compare indole analogues that differ in their heteroatom (Table 2). The contribution of the N-atom is emphasized when on the basis of initial rates the antioxidant activity of indole is compared with that of indene, benzthiophene and benzofuran. In no case is the analogue comparable with indole, but in accordance with expectations, the sulfur com-

Table 2. *Antioxidant activity of indole and its analogues.*

Compound	Structure	Inhibition at 10^{-4} M (%)
Indole		100
Indene		0
Benzthiophene (thianaphthene) . . .		0 ¹
2,3 Benzofuran		0

¹ 15 % at 10^{-3} M.

pound has a low level activity, suggesting its participation as a reducing agent rather than an antioxidant.

Although they have not attracted the attention of the plant physiologist, the β -phenylethylamines, relatives of tyrosine, include compounds of considerable biological importance. The few phenylethylamines which have been tested are noted here entirely for the sake of comparison: On the basis of initial rates, at 10^{-4} M, tyramine and thyroxine inhibited iodide oxidation about 50 %; Mescaline and diiodothyronine inhibited 10—25 %.

Thus far, all compounds tested have included an aromatic group. Although aromaticity may be of great importance physiologically, and of significance with respect to chemical activity, the evaluation of simpler aliphatic amines was called for. In general aliphatic amines are far less active than indoles, but possess inhibitory ability, even when fully substituted as in triethylamine.

At 10^{-4} M, di-n-propylamine and triethylamine had no effect on initial rate of oxidation; at 10^{-3} M slight activity; and at 10^{-2} M, 75 % or more inhibition suggesting again that they serve only as weak reducing agents. Glucoseamine, at 10^{-4} M inhibited (iodide oxidation) slightly (10 %) and ethylenediamine tetraacetic acid (EDTA) inhibited about 5 % at 10^{-4} M.

The occurrence of antioxidant activity among the indoles phenylethylamines, and alkylamines suggested in turn the hydrazines as additional possible antioxidants. Further, implication of N(III) as an important factor in the activity of organic compounds, renders hydroxylamine and hydrazine valuable test substances.

Table 3. Antioxidant properties of hydrazine and related molecules.

Compound	Inhibition (%, 1 st 2 min)	Concentra- tion	Lag period (min)	Rate—1 st 2 min after lag (% control rate)
Semicarbazide	48	10^{-5}	0	—
	100	10^{-4}	0	—
Hydroxylamine	42	10^{-5}	2	47
	100	10^{-4}	8	20
Hydrazine	100	10^{-5}	6	10
	100	10^{-4}	17	< 1
Benzoylhydrazine	100	10^{-5}	4	72
	100	10^{-4}	14	4
Isonicotinic acid hydrazide	100	10^{-5}	4	74
	100	10^{-4}	14	4

The present data (Table 3) show that hydrazine and all related compounds tested are indeed powerful inhibitors of I^- peroxidation. Sizable inhibitions are encountered with 10^{-5} M concentrations (H_2O_2 /inhibitor=100). Semicarbazide, and hydroxylamine are lower in activity than the other three compounds. The action of hydrazine extends over a lag period of considerable duration; substitution of benzoyl- or isonicotinoyl groups reduces the effective period of complete inhibitions.

2. *Sulfide oxidation.* The oxidation of $S^{=}$ to $SO_3^{=}$ by H_2O_2 was selected as an additional test system. It was not intended that a full compound-by-compound comparison be made, but that the existence of the antioxidant properties under conditions differing markedly from those of the iodide system be evaluated.

Oxidation of sulfide was followed by loss of the characteristic 229 m μ absorption peak. The Beckman DK-II Recording Spectrophotometer was used. The absorbency of Na_2S (pH 9.5) was a linear function of concentration from 0—200 μ moles/L; the reaction mixture was set up with 200 μ moles/L of Na_2S and 600 μ moles/L of H_2O_2 . Without added catalyst, and at 25°C, the time course of oxidation was linear until about half the initial sulfide had been consumed.

Inhibitors were necessarily employed at concentrations sufficiently low to eliminate their own ultraviolet absorption. After preliminary trials, a concentration of 10 μ moles/L was selected, corresponding to a H_2O_2 /inhibitor ratio of 60 : 1.

The six inhibitors tested were all active (Table 4), and all but one of them yielded initial lag periods (as shown on Figure 3 for the iodide system), including notably IAA and skatole (3-methylindole), which had not exhibited lags with iodide. Indole and hydrazine although potent in the iodide system

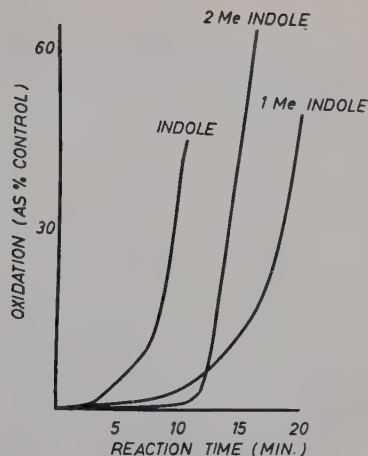


Figure 3. Examples of inhibitor of the second type showing lag period.

were weak inhibitors (10—12 %). Skatole, IAA, mescaline and thyroxin were active to about the same extent in the respective oxidation systems.

The range of conditions, therefore, under which the compounds studied can regulate oxidations includes the following: (a) air oxidations of pyrogallol in various alcohols (27); (b) $\text{Fe}^{3\pm}$ catalyzed peroxidation of I^- in ethanol; (c) peroxidation of I^- in weakly acidic aqueous media; (d) peroxidation of $\text{S}^=$ in alkaline aqueous media.

3. *Other systems.* The Fe(III) catalyzed peroxidations of benzidine and o-phenylene diamine were observed to be retarded by IAA. The experiments employed substrates at 10^{-2} M, H_2O_2 at 10^{-2} M, Fe at 10^{-5} M and noticeable retardations were obtained with IAA at 10^{-3} M.

Peroxidation of pyrogallol (substrate 10^{-3} M, H_2O_2 10^{-2} M pH 4.5—6.5) in disks of potato tuber (2—4 mm thickness) was inhibited by 10^{-4} M hydrazine and 10^{-3} M IAA.

Table 4. Inhibition of sulfide oxidation. All inhibitors at 10^{-5} M.

Compound	Inhibition (% 1 st 2 min)	Lag period (min)	Rate—1 st 2 min after lag (% of control)
3-methylindole	53	10	25
Indole-3-acetic acid.	33	5	45
Indole	12	4	51
Mescaline	11	< 1	80
Hydrazine	10	1	88
Thyroxin	50	10	32

Reference has already been made to inhibition of the enzymic peroxidation of pyrogallol and eugenol by IAA and hydrazine as well as more commonly recognized oxidation inhibitors such as ascorbic acid, cysteine, and guaiacol (26).

III. Physiological Activity of N-containing Antioxidants

1. *Antioxidant properties of known biological agents.* Although in some proposed mechanisms of phytohormone action auxins have been associated with ascorbic acid or the ascorbic acid-regulated balance between thiols and their disulfides (3), the direct participation of growth regulators as antioxidants has not hitherto been suggested.

It is of interest that indole, an active antioxidant, exhibits some auxin activity whereas oxindoles, inactive as antioxidants, are also devoid of auxin activity (31). Thimann, who studied the foregoing and other compounds, has recognized that structural requirements for auxin activity are not well described by existing theories relating structure to growth-regulating properties. IAA itself can act both as a growth-promotor and growth-inhibitor depending upon test species, test organ or process, experimental environment, and experimental technique. It would, therefore, be naive to expect a correlation between antioxidant properties and a single direction or mode of biological response. On the other hand, it is chemically and physiologically reasonable to expect some kind of biological activity to be associated with the antioxidant property. Thus, 5-hydroxytryptamine (serotonin) has been reported to be a root growth inhibitor, and the related 5-hydroxy-IAA exhibits weak activity in the avena test (3). Serotonin is, of course, recognized as an important psychodynamic agent (34, 36), and both compounds are highly active as antioxidants. Tryptamine, itself, is a sympathomimetic substance which has also been found to protect mice against oxygen poisoning (14). Mescaline is known as a psychogenic agent (22, 35); tyramine acts upon the mammalian vascular system. Thyroxine and diiodothyronine are mammalian hormones. Thyroxine is known to protect mitochondria against oxygen damage (21) and to increase the SH/S-S ratio in mammals (18).

EDTA has been reported to possess weak auxin activity (20, 33), to protect DNA against the effect of high oxygen pressure and to prevent the oxidation of glutathione (17). Although it is natural to associate effect of EDTA with its chelating properties, Gilbert *et al.* have found reason to question this role in connection with their work on glutathione oxidation, and in the iodide

Table 5. *Promotion of germination by isoniazid.*

Plant material	Incubation Time (Hrs)	Control	10^{-4} M Isoniazid
Turnip	16	0%	0%
	18	1	22
	20	3	45
	24	16	91
	30	73	97
Rice	30	0	0
	32	4	16
	36	10	54
	40	15	65
	48	28	68

oxidation system, EDTA has been established as an antioxidant, revealing a parallelism in biological and chemical action to IAA.

Hydrazine and related compounds are also noted for their biological activity, principally as metabolic poisons (hydrazine, hydroxylamine) and also as chemotherapeutic and psychodynamic agents (isoniazid and other substituted hydrazines). As an antioxidant, hydrazine exhibited twice the activity of hydroxylamine. It is perhaps of significance that this 2 : 1 relationship applies also to the inhibition by these compounds of bacterial diamineoxidase and to their relative tuberculostatic properties (23).

2. *Responses of seeds to antioxidant treatment:* Germination and early seedling development were chosen as processes for initial study in connection with antioxidants surveyed chemically. Species selected were turnip, *Brassica rapa* var. Purple Top White Globe; lettuce, *Lactuca sativa* var. Black Seeded Simpson; and rice, *Oryza sativa* var. Blue Bonnet. All seeds were of the 1958 commercial crop. Germination was carried out in Petri dishes on moist filter paper at 24–25°C. Seeds were not presoaked, but were maintained in M/15 phosphate buffer pH 6.65, with or without added compounds to be tested. Germination was usually carried on in dim fluorescent light, about 10 ft candles.

Germination percentages were based upon 100 or more seeds as the aggregate of several replicate experiments. Growth data were based on measurements of 50–60 seedlings.

The time course for turnip germination was strikingly altered by 10^{-4} M isonicotinic acid hydrazide (table 5). Substantial germination (about 22 %) had occurred in the treated group after 18 hours incubation at which time controls averaged only about 1 %. Germination responses of rice to isoniazid were also of considerable magnitude and Indole-3-ethylamine at 10^{-4} M was essentially identical in activity with the hydrazide.

Table 6. *Growth characteristics of seedlings cultured in antioxidant solutions.*

Species	Organ	Compound ¹	Response (Control = 100)
Turnip	Radicle	Isonicotinic acid hydrazide	300 (28) ²
		Indole-3-ethylamine.....	600 (42)
		Hydrazine	350 (42)
		Skatole	140 (42)
Rice	Radicle	Skatole	203 (100)
		Isonicotinic acid hydrazide	278 (83)
		Isonicotinic acid hydrazide	516 (83)
		Indole-3-ethylamine.....	263 (83)
	Coleoptile	Indole-3-ethylamine.....	533 (83)
		Isonicotinic acid hydrazide	

¹ Hydrazine 10^{-7} M, skatole 10^{-6} M, others 10^{-4} M.

² Number refers to hours culture 22–24°C.

The germination of lettuce was hastened by hydrazine at concentrations of about 5×10^{-10} M whereas the germination of turnip was not appreciably accelerated by this compound at concentrations below 10^{-7} M. In both cases the maximum effect obtained early in the incubation period was of the order of 300 %. Concentrations of hydrazine in excess of 10^{-5} M were progressively more inhibitory.

Observations of seedling responses with turnip and rice (Table 6) demonstrate the activity of hydrazine, ioniazid, skatole and tryptamine in promoting both root and coleoptile growth. Growth promotion of seven additional indoles and hydrazines, all of high antioxidant activity, is currently under examination and serves to support the pictured relationship between chemical and physiological properties.

IV. Conclusions

Antioxidant activity is a common feature of many organic N-compounds, including biologically active substances and their relatives. In some instances this activity has been implicated in biological systems albeit under experimental conditions, as for example, in the protection of organisms, organelles or bio-organic compounds against oxidation. In the light of increasing knowledge about oxygen toxicity, it is not unreasonable to view the antioxidant property as a feature of potentially great physiological importance. Furthermore, it is difficult to disregard this property among hormonal substances when their mode of action is under consideration. It cannot be proven on present evidence whether the antioxidant property contributes in small or great measure to biological activity. Nevertheless, it is reasonable on chemical

grounds to expect the antioxidant function to be of considerable importance in cellular systems with their abundance of oxidizable molecules and active electron transport chains. Hence among the many effects of natural and synthetic compounds on growth it is to be expected that some will be shown to arise from the action of specific, intracellularly localized antioxidants.

Summary

1. General considerations on oxygen toxicity suggest an important role for antioxidants as protectants for the more labile cellular components, including proteins and DNA.
2. Low effective levels of protectants against experimental oxygen poisoning suggest radical chain-breaking (model: NO) or self-regenerating (model: $\text{Co}^{2+} \rightarrow \text{Co}^{3+}$) properties for these compounds.
3. Consideration of the elements of common biological occurrence suggests N (and possibly S) compounds as potentially important antioxidants. It is suggested that the unshared electron pair of N(III) is of importance in the activity of organic N-compounds against oxidants or oxidizing radicals.
4. Several chemical oxidations and a variety of conditions have been used in demonstrating the antioxidant activity of IAA and a variety of indoles, hydrazines and other N-compounds. Test systems include pyrogallol — O_2 , S^{2-} — H_2O_2 and I^- — H_2O_2 , especially the last. Reaction media range from acidic to alkaline and include both aqueous and ethanolic systems.
5. Among the indoles, antioxidant activity can be related to structure. Their ability to decrease the initial rate of I^- oxidation is reduced by: (a) substitution at the 3-position; (b) introduction of an electron-withdrawing group at the 2- or 3-position and (c) replacement of N with another atom or heteroatom. This kind of activity may be increased however by: (a) introduction of OH into the benzene ring and (b) by introduction of an electron-releasing agent at the 2-position. Activity is not affected by (a) substitution on N, or (b) 3-substitution with an ethylamine group.
6. Activity among other chemical groups has not yet been related to structure, but several biologically active compounds including thyroxine and isoniazid are powerful antioxidants. Even simple aliphatic amines, including a tertiary amine, exhibit weak inhibiting properties.
7. A number of the active compounds are known to have some kind of biological activity at comparatively low concentrations, including serotonin, 5-hydroxy-IAA, indole and EDTA.
8. Experiments carried out on germination and early seedling growth with

- turnip, rice and lettuce show isoniazid, hydrazine, indole-3-ethylamine, and skatole, all powerful antioxidants, to be growth promoters as well.
9. It is concluded that although definite "cause-effect" relationships between antioxidative and biological activities have not yet been proven, the widespread occurrence of antioxidants together with our current information on oxygen toxicity calls for serious consideration when mechanism of hormone and regulator actions are under consideration.

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Factors Affecting Light Response in Phototactic Algae

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Most motile algae react to light by swimming either toward the light source (positive phototaxis) or away from it (negative phototaxis). In some cases a non-phototactic reaction occurs (in this paper termed random motion). A distinction is usually made between topo-phototaxis, which is a swimming according to the direction of the light which occurs in motile algae and zoospores and phobo-phototaxis, which occurs in purple bacteria and possibly in some algae (for review of the literature see Haupt 1958). In this investigation no attempt has been made to distinguish between these two different types. The term phototaxis is here used for orientation and accumulation according to a light source.

A reversal from positive to negative phototaxis and *vice versa*, and a random motion situation have been induced in different organisms by a number of factors (light intensity, ion concentration and ion balance, pH, and total and partial gas pressure). The literature and results from such experiments are covered in the following papers: Mast 1932, Luntz 1932, Mainx *et al.* 1940, and Haupt 1959. Results from these earlier investigations are often contradictory. It has therefore not been possible to give a unified explanation for the phototactic behaviour in algae, based upon environmental and/or internal factors. At the start of this investigation some experiments were performed in order to test the effect of different factors upon our particular material (Dinophyceae and Volvocales).

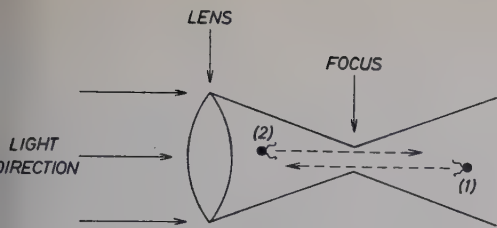


Figure 1.

Figure 1. The behaviour of a positively phototactic organism (1) and a negatively phototactic organism (2) in a focused beam. Light intensity in focus $\sim 200,000$ Lux. For explanation see text.

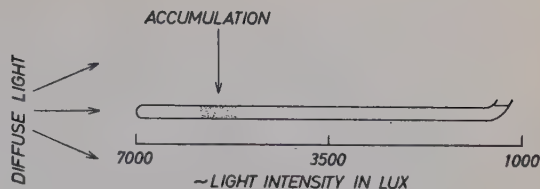


Figure 2.

Figure 2. The accumulation of phototactic organisms in long-time experiments in properly balanced medium, e.g., sea water. For explanation see text.

Results

1. Light Intensity

The following organisms have been subjected to analysis: Dinophyceae: *Goniaulax catenella* Whedon & Kofoid (Halldal's strain), *Goniaulax polyedra* Stein, *Amphidinium* sp. *Exuviaella baltica* Lohmann, *Peridinium trochoideum* (Stein) Lemmermann, *Prorocentrum micans* Ehrenberg. All except *G. catenella* from the culture collection of Institute for Marine Biology, University of Oslo. Volvocales: *Dunaliella salina* (Dunal) Teodoresco (Gibor's strain), *Dunaliella viridis* Teodoresco (Gibor's strain), *Dunaliella* cf. *euchlora* Lerche (Halldal's strain), *Platymonas subcordiformis* (Wille) Hazen (Gibor's strain), *Stephanoptera gracilis* (Artari) Smith (Gibor's strain). All have been grown in Erdschreiber solution (Föyn 1934).

The phototactic behaviour of these organisms was studied in diffuse light, in a columnated beam and in a focused beam. The same general conclusion has been drawn for all the species and is as follows: The light intensity is primarily not responsible for the alternation between positive and negative phototaxis. A group of organisms with a particular phototactic response can not be altered in their reaction by increasing or decreasing the light intensity. In short-time experiments (from 2 to 15 minutes) positively reacting organisms will swim toward the light, and negatively reacting organisms will swim away from the light at all light intensities. This phototactic behaviour can be demonstrated as illustrated in Figure 1. The experiment was suggested by Dr. J. B. Thomas and co-workers at the Physical Institute of the State University, Utrecht, the Netherlands. In a focused beam an organism outside the focus showing positive phototaxis, Figure 1 (1), will swim toward the light source to increasing light intensities, pass the focus and continue toward the light to lower light intensities. Similarly an organism between the focus and the light source showing negative phototaxis (2) will swim away from

the light source, but toward increasing light intensities, pass the focus and continue in a negative direction.

In a medium with a suitable salt balance and pH, *e.g.* Erdschreiber, the light intensity may affect phototaxis secondarily in that presumably algal photosynthesis either alters the environmental factors, salt balance, pH, or the physiology of the algae. Under such conditions the algae will gather at a certain light intensity and stay there for hours, and sometimes up to several days. With fluorescent light the accumulation ordinarily occurs at about 5000 to 6000 Lux (Figure 2), and the accumulation is in the course of a few days shifted to about 3000 to 4000 Lux. *Prorocentrum micans*, however, accumulates at the highest intensity (7000 Lux). Nordli (1958) reports accumulation of *Ceratia* at the highest intensity (7000 Lux) for most species, while *C. tripos* showed no accumulation.

2. Total and Partial Gas Pressure in the Medium

Only *Platymonas subcordiformis* has been subject to analysis for the possible effect of changes in gas pressure in the medium. For these experiments *Platymonas* was grown in the following medium (Medium I): NaCl 0.5 M; MgCl₂ 0.025 M; KCl 0.01 M; CaCl₂ 0.005 M; Na₂HPO₄ $2 \cdot 10^{-4}$ M; NaNO₃ $2 \cdot 10^{-3}$ M; trace elements according to Hutner and Provasoli (1951 p. 101) pH 5.7–6.0.

No change in phototactic response was observed when cultures of *Platymonas* were bubbled through with different gases (air; 5 % CO₂ in air; N₂; or H₂). The phototactic behaviour was also studied in the absence of air in the following way: Two filtering flasks, one containing positively reacting organisms (one-week-old culture) the other negatively reacting organisms (newly transferred) were connected to a water suction pump and the air was continuously evacuated for 6 hours at 40 mm Hg pressure. No change in response was observed after such treatment, except that the negatively reacting organisms at this time turned non-phototactic, which presumably was the ordinary transition from negative to positive phototaxis that usually occurs in Medium I at this time after inoculation. These experiments and the whole experience in culturing *Platymonas* indicate that the total and partial gas pressure in the medium is not responsible for the change from positive to negative reaction and *vice versa* and that O₂ and CO₂ in the medium are not needed for motion. Different checks on the other Volvocales have given the same results. Blum and Fox (1932) also tried the response of positively reacting *Dunaliella salina* in the absence of air and recorded no change in phototactic response or activity.

3. Necessary Ions and Ion Balances

Preliminary reports on this have been given (Halldal 1956, 1957).

Material and methods. *Platymonas subcordiformis* was grown in Medium I in light from fluorescent tubes, incandescent lamps, or diffuse daylight. Cultures grown in diffuse daylight are most suited for phototactic experiments and these have been used in most cases. The organisms were either concentrated by their phototaxis (earlier experiments) or more preferably concentrated by centrifugation at 500 *g* for five minutes (later experiments). One or two drops of the concentrated *Platymonas* were then added to 10 ml of the solution where its response was to be tested. After inoculation the resuspended organisms were shaken for five minutes and 1 ml of the suspension was transferred to a watch-glass and the reaction studied under low power in a microscope. A 40 W 220 V microscope lamp fitted with a blue filter (light intensity ~ 4000 Lux) served as light source both for phototactic stimulation and microscope illumination. The cells were first accumulated by their phototaxis (if any), then the watch-glass was turned around and the swimming rate was recorded in timing individual cells passing a known distance and averaging the results. The swimming rate is very uniform within one and the same sample and only a few (10 to 20) readings are needed to bring the accuracy to the desired level (relative error $\pm 5\%$). In long-time experiments the resuspension of the organisms was performed in Erlenmeyer flasks which were either placed in the dark or in the light and the behaviour of the organisms was studied at intervals by transferring 1 ml to a watch-glass.

The motility and phototactic activity of *Platymonas* have been analyzed at different concentrations for all the major ions of Medium I. The cells were motionless in the absence of both Ca^{++} and Mg^{++} , and the motility, phototactic activity and the mode of response were greatly influenced by K^+ , but K^+ alone did not support motion. With traces of K^+ (stored in the cells or as "contamination" from the inoculum) Ca^{++} causes a negative and Mg^{++} a positive phototaxis. The effect of Ca^{++} was measured as the negative phototactic activity, recorded as swimming rate, obtained with different concentrations of CaCl_2 ; the effect of Mg^{++} was measured as the positive phototactic activity, recorded as swimming rate, obtained with different concentrations of MgCl_2 (Figure 3). The ionic strength was adjusted to $1.0\ \mu$ with NaCl (above $0.333\ M$ MgCl_2 in pure MgCl_2 solution). At Ca^{++} , Mg^{++} , and K^+ concentrations favourable for phototaxis, ionic strength values between 0.5 and $1.3\ \mu$ adjusted with NaCl have very little effect on the reaction. In different experiments the ionic strength has therefore been kept at a value within this interval, and in one and the same experiment the ionic strength has been kept constant (if possible).

The cells showed no motion at a Ca^{++} concentration below $0.002\ M$ (the exact lower limit has not been determined). The cells moved at these low

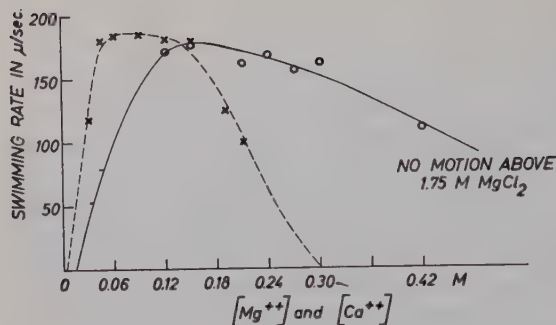


Figure 3. The positive phototactic activity in *Platymonas* obtained with different MgCl_2 concentration (solid line), and the negative phototactic activity obtained with different CaCl_2 concentrations (dashed line). Ionic strength adjusted to 1.0μ with NaCl (above 0.333 M MgCl_2 in pure MgCl_2 solution) pH 5.8, temperature 22°C , light intensity $\sim 4000 \text{ Lux}$.

concentrations but showed very little phototaxis. The negative phototactic activity has an optimum at 0.04 to 0.1 M . Between 0.15 and 0.3 M Ca^{++} the cells moved but showed very little phototaxis. No motion was observed above 0.3 M Ca^{++} .

For Mg^{++} the cells were motionless somewhat below 0.01 M (the exact lower limit has not been determined). The positive phototactic activity has an optimum around 0.1 to 0.15 M Mg^{++} . Between 0.3 and 1.75 M Mg^{++} the cells moved but showed very little phototaxis. The cells were motionless at higher Mg^{++} concentrations.

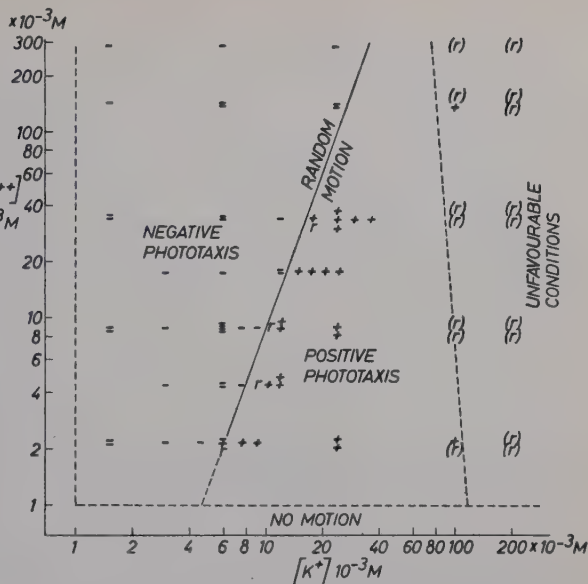
At Ca^{++} concentrations between 0.01 and 0.04 M , and Mg^{++} concentrations between 0.07 and 0.3 M (ionic strength 1.0 , pH 5.8) the cells showed random motion at a $\text{Ca}^{++} : \text{Mg}^{++}$ ratio $= 1 : 6$ (Halldal 1957).

These were short-time experiments. The motility ceased after about 15 to 30 minutes in CaCl_2 solutions and was very much reduced in MgCl_2 (see below). In the presence of proper concentrations of K^+ the motility and phototactic activity continued for several days.

4. $\text{K}^+ - \text{Ca}^{++}$ Interaction

The effect of different $\text{Ca}^{++} : \text{K}^+$ ratios upon phototaxis has been studied in long-time experiments at CaCl_2 concentrations between 0.002 M and 0.288 M , and at KCl concentrations between 0.0015 M and 0.2 M . The resuspended cells were kept in the solution where the reaction was to be studied for one hour for adaptation in diffuse daylight at about 1000 Lux . The response was then recorded at different time intervals during the next two days. The result is given in Figure 4. The cells were motionless at K^+ concentrations somewhere below 0.0015 M independent of Ca^{++} concentration, and likewise motionless at Ca^{++} concentrations below 0.002 M independent of K^+ concentration. At a certain $\text{K}^+ - \text{Ca}^{++}$ balance a random

Figure 4. The K^+ — Ca^{++} interaction upon the motility and phototactic behaviour of *Platyomonas*. Ionic strength adjusted to 0.5μ with NaCl, pH 7.5 with Veronal-Na/HCl, light intensity ~ 4000 Lux, + observed positive phototaxis, — observed negative phototaxis, r random motion at favourable conditions, (r) random motion at unfavourable conditions. Repeated symbols observations from different experiments.



motion situation was obtained. This random motion balance has been calculated to follow the empirical formula:

$$[Ca^{++}] = 0.015 \cdot [K^+]^{2.7}$$

$[Ca^{++}]$ and $[K^+]$ in $10^{-3} M$ and Ca^{++} concentrations between $2.2 \cdot 10^{-3}$ and $140 \cdot 10^{-3} M$, and K^+ concentrations between $5 \cdot 10^{-3}$ and $30 \cdot 10^{-3} M$. Positive phototaxis occurred at a relative concentration of Ca^{++} lower than that given by this formula, and negative phototaxis at a higher Ca^{++} concentration. The random motion situation occurred consistently at the random motion line of Figure 4 with only small deviations. It was not possible to alter the response by placing the cells in the dark for 24 hours, or at different light intensities. Further analysis on the random motion situation caused by the K^+ — Ca^{++} balance is on the program.

Above $0.2 M Ca^{++}$, and $0.1 M K^+$ the conditions were unfavourable for motion and phototaxis. The cells moved but showed little if any phototaxis.

5. K^+ — Mg^{++} Interaction

The effect of different $K^+ : Mg^{++}$ ratios upon phototaxis has been studied in long-time experiments at KCl concentrations between zero and $0.08 M$, and $MgCl_2$ concentrations between 0.0045 and $0.4 M$. The same experimental procedure as described for the K^+ — Ca^{++} interaction has been followed. The

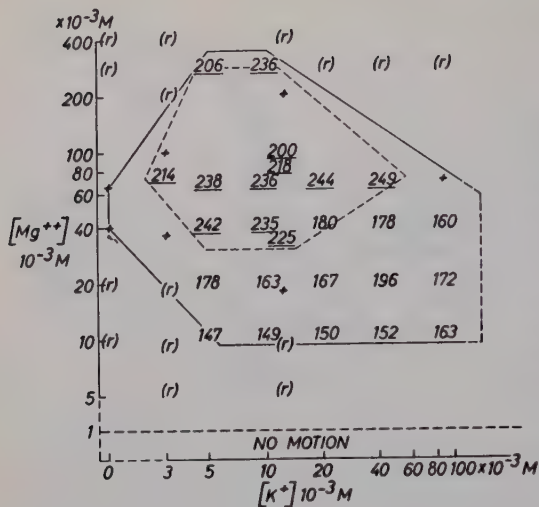


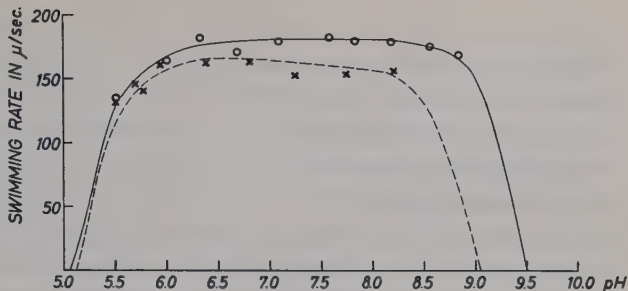
Figure 5. The K^+ — Mg^{++} interaction upon the motility and phototactic behaviour of *Platymonas*. Ionic strength adjusted to 1.0μ with NaCl, pH 7.5 with Veronal-Na/HCl, temperature 22°C , light intensity ~ 4000 Lux. The figures denote swimming rate in μ/sec in positive direction, + observed faint positive phototaxis, (r) random motion at unfavourable conditions, the region with positive phototaxis has been framed, the optimal region for positive phototaxis lies within the inner dotted frame.

result is given in Figure 5. At all the K — Mg ion combinations where the cells move the reaction was positive, except at high concentrations where random motion was the result of unfavourable conditions. No motion occurred in the absence of Mg^{++} , while a very reduced motion occurred in the absence of K^+ with a slight positive phototaxis at 0.04 to $0.07 M$ Mg^{++} .

6. pH Activity Curves

The pH activity curves for both positive and negative phototaxis have been determined. Positive phototaxis was produced by $0.1 M$ Mg^{++} and traces of K^+ , and negative phototaxis with $0.05 M$ Ca^{++} and traces of K^+ . The results are given in Figure 6. No motion occurs below pH ca. 5.2. The positive phototactic activity is fairly constant and close to optimum between pH 6.0 and 9.0 and drops to zero at somewhat above pH 9.5. Above this value no motion occurs. The negative phototactic activity is fairly constant and close to optimum between pH 6.0 and 8.7 and drops to zero at pH 9.0. Above this value no motion occurred. The pH activity curves for positive and negative phototaxis have also been checked in buffered sea-water from pH 5.0 to pH 10.0 (pH 5.0—6.8 KH-phthalate/NaOH; pH 7.2—10 Veronal-Na/HCl). Negative phototaxis was obtained with newly transferred *Platymonas*, and positive phototaxis in the same solutions the next day. The pH activity curves for both positive and negative phototaxis followed the pH activity curve for positive phototaxis of Figure 6.

Figure 6. The pH activity curve for positive phototaxis (continuous line) obtained with 0.1 M $MgCl_2$, and negative phototaxis (dashed curve) obtained with 0.05 M $CaCl_2$. Both with traces of K^+ , pH 5.0 to 6.5 KH-phthalate/NaOH, pH 7.0–10 Boric acid. Ionic strength 1.0μ with NaCl, temperature $\sim 22^\circ C$, light intensity ~ 4000 Lux.



Conclusions

The experiments were carried out to investigate the environmental factors affecting the reversal from positive to negative phototaxis, and also under which conditions phototaxis occurs. When motile organisms are cultured in the laboratory under constant light conditions, it is observed that they sometimes gather nearest the light source, at other times farthest away from it, and that they also may be clustered at certain regions within the liquid. This well-known experience suggests that light intensity alone is not decisive for the phototactic behaviour, but that light, presumably through the photosynthesis of the algae, may induce changes in the environmental conditions or may affect the physiology of the algae which in turn influences phototaxis.

The results from the light intensity—light direction experiments (Figures 1 and 2) show that it is necessary to differentiate between short-time and long-time experiments. In short-time experiments cells with a certain phototactic response move according to the direction of the light independent of the light intensity. In long-time experiments in a properly balanced medium, *e.g.*, sea water, the cells after some time will gather at a certain intensity and stay there. If this situation is to be obtained, it is essential that the medium is properly balanced for Mg^{++} , Ca^{++} , and K^+ and that the concentrations of these ions are suitable for phototaxis. In the absence of Ca^{++} positive phototaxis occurs at all $K^+ : Mg^{++}$ ratios. In the absence of Mg^{++} , presence of Ca^{++} and at low K^+ concentration negative phototaxis occurs, and at higher K^+ concentrations positive phototaxis is the result (Figure 3). These specific modes of reactions are independent of light intensity and preillumination. Positive and negative reactions have also been produced with different $Ca^{++} : Mg^{++}$ ratios (Halldal, 1957).

Specimens of sperm flagella vibrate in the presence of adenosinetriphosphate (ATP) (Hoffmann-Berling 1955), and it has earlier been assumed that the driving power in the flagella is an ATP-motor (Links 1955). The rhythmic bending which is the result of contractions and relaxation on both

sides of the flagella presumably involves ATP, Ca^{++} , Mg^{++} , and K^+ . In *Platymonas* the flagella move either in the presence of a Mg^{++} — K^+ combination, or in the presence of Ca^{++} and K^+ . No attempt will be made to fit these observations into any of the theories about muscle contraction and relaxation. However, our observations concerning phototaxis will be given some attention.

One may assume that in phototaxis some factor must be in a proper balance when the random motion situation is obtained. This factor may be a proper balance between ATP supplied, and ATP consumed as the result of the ATPase activity within the flagellar apparatus. This balance may be regulated with coarse, fine, and micro adjustments. (1) A coarse adjustment is obtained with the ions Mg^{++} , Ca^{++} , and K^+ , or with the ion pairs Ca^{++} — K^+ ; Ca^{++} — Mg^{++} . With combinations of these ions far from the random motion line of Figure 3 and far from Ca^{++} ; Mg^{++} ratios 1 : 6 (Halldal 1957), a change in the mode of reaction cannot be induced by illumination. (2) A fine adjustment is obtained with light when the medium is properly balanced for the ions Mg^{++} , Ca^{++} , and K^+ , *e.g.*, in sea water and Medium I. Algal photosynthesis may in this case induce changes either directly in the physiology of the algae, or indirectly by altering environmental conditions. Another factor which may induce changes in this situation is changes in phosphorylation. Preliminary experiments with 2,4-dinitrophenol (DNP), which in low concentrations uncouples phosphorylation without inhibiting oxidation or photolysis of water, have been performed on negatively reacting *Platymonas* in sea water (unpublished data). At concentrations above 10^{-3} M DNP the reaction was immediately reversed to positive. After three hours positive reaction occurred at concentrations above $5 \cdot 10^{-4}$ M DNP, and negative reaction at lower concentrations. Phosphorylation may in fact be a "coarse" adjustment in phototaxis. (3) Finally it is assumed that light directly serves as a "microbalance" in phototaxis by a photochemical reaction through the pigment (pigments) involved in phototaxis. This last assumption is based upon the experience with *Platymonas* in well-balanced medium showing either random motion or a slight positive reaction. When the conditions in such cultures are slightly altered by pouring the culture into a new container (no medium) the reaction is swiftly changed to negative. After a few minutes the original situation is re-established. In positive phototaxis *Platymonas* orients presumably in such a way that the photosensitive spot is maximally illuminated, and in negative reaction it is shaded by the cell (Halldal, 1958). The nature of this (these) pigment is unknown and nothing is known about the photochemical reaction involved and what it influences. We may just guess that it has something to do with flagellar ATP balance, or ATPase activity.

This investigation must be considered as a survey. It is now possible to control phototaxis by the Mg^{++} , Ca^{++} , K^+ balance, and with the aid of differently reacting organisms it should be possible to analyze more thoroughly the effect of different isolated reactions upon phototaxis, as, for example, oxidative and photo phosphorylation, direct and indirect effect of photosynthesis, minor changes in pH, and the direct effect of light upon the pigment involved in phototaxis.

Finally, it should be stated that these results apply primarily to *Platymonas subcordiformis*, which is very suited for experiments on phototaxis. Some of the results have been checked on other salt water forms. It has, however, not been possible to repeat these experiments using *Chlamydomonas moewusii* (+ and —) obtained from Dr. Fogg. The brine flagellate *Platymonas subcordiformis* lives in high salt concentration, and it is therefore possible to investigate the effect of different ions by the use of sort of "expanded scales" compared with ion concentrations of fresh water. *Chlamydomonas* showed motion and pronounced positive phototaxis after repeated centrifugations and resuspensions in distilled water, evidently utilizing ions stored in the cells. *Platymonas*, on the other hand, is motionless when inoculated in a medium lacking both Mg^{++} and Ca^{++} .

Summary

A reversal from positive to negative phototaxis and *vice versa*, and a random motion situation occurred in all the species examined (salt water Volvocales and Dinophyceae). The light intensity is primarily not responsible for changes in phototactic response, but light may affect phototaxis secondarily through algal photosynthesis which presumably either alters the physiology of the algae or environmental conditions. Experiments with *Platymonas* show that changes in CO_2 , O_2 or total gas pressure in the medium do not affect phototactic response. Mg^{++} and Ca^{++} are needed for motion, and the motility, the phototactic activity and the mode of response are influenced by K^+ and by the balance between these three ions. The pH activity curves for motility and positive and negative phototaxis have a broad maximum between pH 6.5 and 8.5. No motion occurs below pH 5.2 and above pH 9.5.

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Neomorphosis in Seed Plants Induced by Amino Acids I. *Oenanthe aquatica*

By

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It was reported in brief (Waris 1957 a) that a fundamental morphological change, without any irreversible change of the genome, took place in *Oenanthe aquatica* when grown in the presence of excess glycine. In this way a new type of plant, called a neomorph, was produced in which differentiation is so reduced that even the family characters are hardly recognizable. Chemical analysis revealed that the neomorphs contain much larger quantities of free amino acids than the normal plants (Miettinen and Waris 1958). Changes of the same kind were subsequently induced with amino acids in *Oenanthe lachenalii* and *Daucus carota* (Waris 1957 b), and have recently been obtained in *Cicuta virosa*. The phenomenon, which has thus proved reproducible, will now be dealt with in greater detail.

Methods

The original cultures were started from seed, but when neomorphs had been obtained these were used to subcultures. Aseptic culture was facilitated by the S-tube flasks, the handling of which has been described in a previous paper (Waris 1958).

The organic substances to be tested were added to a complete mineral solution. The basal mineral solutions mostly employed had the following compositions.

A. In 10 litres: 10 g. KNO_3 , 2 g. $(\text{NH}_4)_2\text{HPO}_4$, 2 g. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 3.3 g. CaCl_2 (anhydrous), 0.25 g. $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.20 g. $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.67 g. disodium ethylenediaminetetraacetic acid (Komplexon III), and 10 ml. of an additional micronutrient solution containing per litre 1.5 g. boric acid, 0.2 g. $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05 g. $\text{CuSO}_4 \cdot$

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5H₂O, 0.05 g. CoSO₄ · 7H₂O, 0.05 g. KJ, and 0.15 g. ammonium molybdate. The pH was adjusted with 0.1 N HCl to about 6.

B. Otherwise as A, but 0.5 g. of the iron salt of EDTA (from Fluka AG.), and the metals Zn, Cu and Co in the form of EDTA chelates (with 2 Na). No additional iron and EDTA.

C. Otherwise as A, but instead of 3.3 g. CaCl₂, a combination of 2 g. CaCl₂ and 1 g. CaCO₃. This solution was used to keep the pH at a higher level, for in the above-mentioned solutions the pH tended to fall as a result of autoclaving and during culture. The initial precipitation dissolved more or less in the long run, depending on growth.

The culture flasks were placed in a window facing south, being thus subjected to varying light and temperature conditions. Fortunately the morphological behaviour of the plants is governed by amino acids to such an extent that the other factors may vary within fairly wide limits without disturbing the results.

The preparations of amino acids (except for glycine), pyrimidine derivatives, beta-phenylethylamine-hydrochloride and ribose were from Hoffmann-La Roche. The sucrose used was an ordinary pure commercial product. The principal mineral substances were of the quality "pro analysi". The water was double-distilled with a Heraeus quartz apparatus provided with an ion filter.

Experimental Results

a) *Growth of Normal Seedlings in Standard Nutrient Solutions.* In the basal mineral solutions, in flask cultures, growth is normal but slow, while in the presence of sucrose it is greatly promoted. At 10 to 60 g. of sucrose per litre the seedlings develop normally if we disregard the fact that the highest concentrations, 50 and 60 g. per litre, are already somewhat inhibitory to growth in size. On the other hand, the higher concentrations, from 30 to 60 g. per litre, favour the production of anthocyanins, to judge from the more or less red areas in the shoots. At the lower concentrations of sucrose, 10 to 30 g. per litre, the seedlings may reach the top of the 21 cm. high flasks, provided with about 250 ml. of nutrient solution, within 3—4 months, the solution becoming filled with the roots, more or less green in colour. Under suitable conditions growth may continue for longer than a year, but eventually the leaves turn yellow, indicating that the conditions have become unsatisfactory, as is to be expected in unchanged solutions.

b) *Induction of Neomorphosis by Glycine.* Although the experiments had been conducted with the intention of inducing morphological changes with amino acids, the course of events could not be anticipated. The phenomenon took place in two flasks, at about 0.1 % and 0.4 % of glycine in the basal solution A with 10 g. of sucrose per litre.

At 0.1 % of glycine one normal seedling was grown, but after developing two cotyledons and a few foliage leaves it ceased growing and turned brown. Within 4 months, in fact, it seemed to be moribund. It was, therefore, a



Figure 1. *Neomorphs* grown at 0.1 % of glycine fill the bottom of the flask, 10 months from the start of the culture. The original seedling will be seen in the middle. Nutrient solution removed. About $\frac{1}{2}$ of the natural size.

Figure 2. *Neomorphs* and the original seedling grown at 0.4 % of glycine.

Figure 3. *Thalloid neomorphs* from the flask in Figure 2. About $\frac{2}{3}$ natural size.

Figure 4. *Seedling-like neomorphs* from the flask in Fig. 2. $\times 3$.



great surprise to find later, within half a year from the start, that minute, fresh green plantlets were present in the flask separated from the original, seemingly dead, seedling. The new plantlets, called neomorphs, originated from minute nodules constricted and detached from the tips of some lateral roots (Figure 5). The detached nodules developed by degrees into embryo-like units showing a distinct polarity with a shoot end and a root end (Figure 8), and eventually into individuals like seedlings at the cotyledon stage (Figure 6, 7), but no further. At all developmental stages the neomorphs could reproduce from outgrowths formed in various parts of them. Within 10 months from the start of the culture the neomorphs filled the volume of the nutrient medium, about 250 ml. in a $\frac{3}{4}$ litre flask (Figure 1).

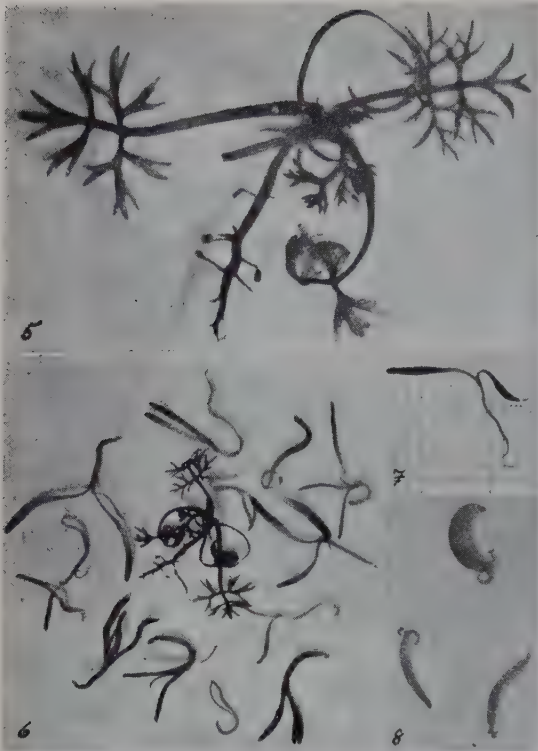


Figure 5. The original seedling from the culture at 0.1 % of glycine, mother plant of the neomorphs, showing constricted root tips. $\times 1.5$.

Figure 6. The same seedling as in Figure 5 and selected neomorphs which resemble cotyledon-stage seedlings. About $\frac{5}{8}$ of the natural size.

Figure 7. A normal seedling. About $\frac{2}{3}$ natural size. Square sides 2.5 mm.

Figure 8. Young neomorphs. $\times 1.8$.

At 0.4 % of glycine neomorphosis took place later and the neomorphs assumed a more irregular, often thalloid shape (Figure 2—4). Their inception from roots was evident, but in this case it is not impossible that a shoot apex might also have contributed to their formation, as it remained alive and showed swollen nodules.

The difference between the two cultures shows that the concentration of amino acid is of great significance. A distinction can be made between neomorphosis-inducing and lower concentrations, though no exact line can be drawn between them. It can be stated that 0.1 % of glycine, corresponding to about 0.0133 *M*, is high enough to induce and to keep up the neomorph pattern of growth in *Oenanthe aquatica* as long as the concentration has not become lowered too much by metabolism. In subcultures at this concentration of glycine, by now during three years, differentiation has become more reduced, the majority of individuals consisting of nodules and embryonic stages.

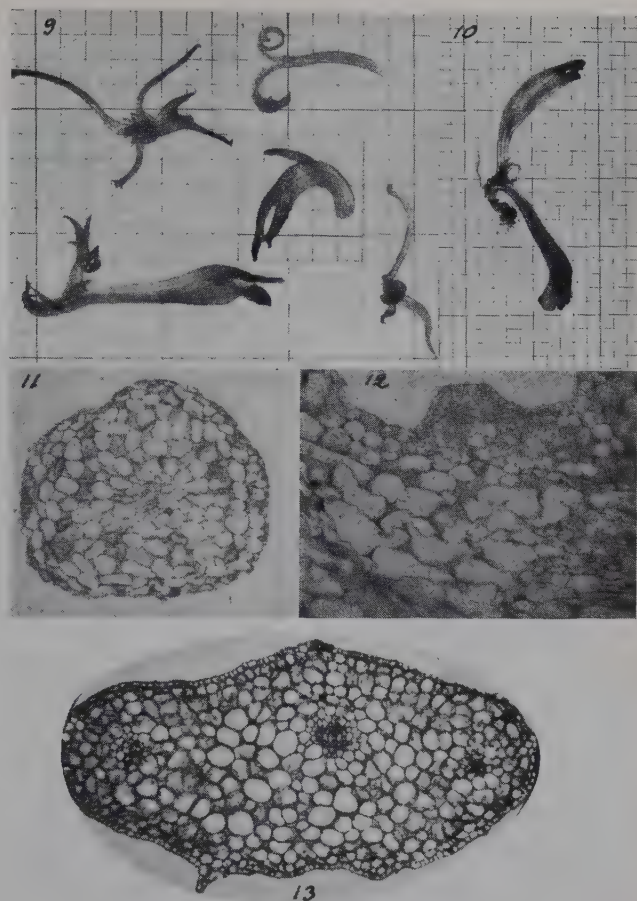
Figure 9. *Neomorphs* grown at 0.01 % of glycine, showing some broad leaves. Square sides 2.5 mm.

Figure 10. A broad-leaved *neomorph*, grown at 0.00063 *M* of arginine-nitrate. Square sides 2.5 mm.

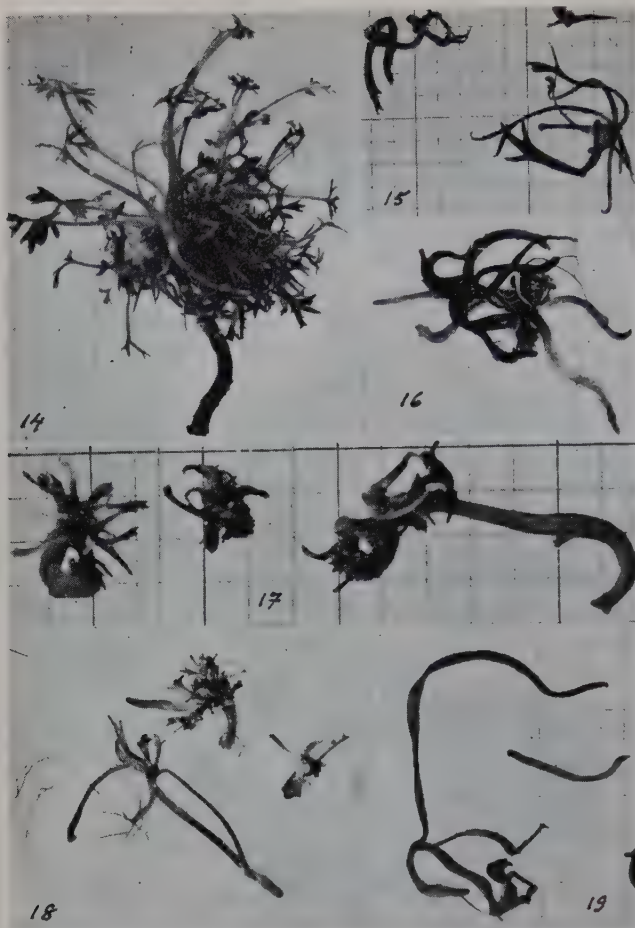
Figure 11. Cross-section of a *neomorph* stem showing a central vascular strand and intercellular spaces filled with organic substances (stained). $\times 45$.

Figure 12. Epidermal outgrowth from a *neomorph*. $\times 110$.

Figure 13. A hand-made cross-section of a semicylindrical *neomorph* leaf. Three vascular strands are to be seen. $\times 45$.



If *neomorphs* are transferred to nutrient solutions containing 0.01 % (about 0.00133 *M*) of glycine, the *neomorph* pattern of growth will be maintained for a few or many months, the individuals becoming larger than at 0.1 %, but eventually some of them will start forming normal shoots. These arise from new growing points, which become recognizable by the formation of minute simple foliage leaves with a few, often three, dents at their tips (as in Figure 18). Subsequently pinnate foliage leaves will be formed. The transition to normal growth is accompanied by the promotion of root growth. At 0.01 % of glycine a broad type of leaf attracted attention which, in certain respects, is intermediate between the *neomorph* and normal leaves, showing several veins (Figure 9). Leaves of the same kind have been seen to occur in cultures with arginine (cf. d, Figure 10).



Figures 14—17. Transition of the neomorphs to normal growth in the presence of 0.0292 M of ribose; photographed 47 days after transfer. 14. Ribose+1 g. of sucrose per litre. 15. Ribose+2 g. of sucrose. 16. Ribose+4 g. of sucrose. 17. Ribose alone. Square sides 2.5 mm.

Figure 18. Transition of the neomorphs to normal growth in a culture grown for 7 months without amino acid but with sucrose. About $\frac{2}{3}$ natural size.

Figure 19. A seedling-like neomorph with long leaves and a leaf-like neomorph from a culture grown for 4 months without amino acid but with sucrose. $\times 0.9$.

c) *Growth of the Neomorphs in the Presence of Sucrose without Amino Acid.* In the basal mineral solutions the neomorphs did not grow well, but if sucrose was present (mostly 10 g. per litre) they grew even better than with amino acid (Figure 19), maintaining the neomorph pattern for several months, but eventually, after 4—5 months, the neomorphs started forming normal shoots, as in the solutions low in glycine. The effect of the changed conditions was first manifested as a promotion of root growth and as an increase in the size of the individuals (Figure 18, 19).

d) *Growth of the Neomorphs with Amino Acids Other than Glycine.* These experiments were made by transferring glycine-induced neomorphs to nutrient solutions containing other amino acids and 10 g. of sucrose per litre. The

basal mineral solutions used were B and in some cases also C, the initial pH varying between 5.2 and 5.9.

With glycine-ethylester-hydrochloride, at 0.001 *M* (pH 5.2), growth was about as good as with glycine, but with the difference that the roots developed somewhat better. Long thin roots appeared within 5 months, and within 9 months normal shoots appeared.

L(+)-arginine, applied as nitrate and as monohydrochloride, showed an effect comparable to glycine but growth seemed somewhat better. Nitrate was applied with the basal solution C in the concentration range 0.00063—0.0013 *M* (initial pH 5.9), monohydrochloride with the basal solution B in the range 0.001—0.004 *M* (pH 5.7). It can be said that the neomorphs developed beautifully in the presence of arginine. For instance, at 0.0013 *M* of arginine nitrate the volume of the nutrient medium, about 250 ml., became filled with neomorphs within 5 months. At 0.00063 *M* growth was equally good, but towards the end of the culture period of 1 1/2 years the neomorphs turned more or less brown, an indication of morbidity. Nevertheless, many of the individuals had started forming aerial shoots with leaves about 2—3 cm. in length, indicating that the concentration of arginine had decreased owing to metabolism. In the presence of arginine, as in the presence of glycine, many of the individuals assumed a seedling-like shape, with the difference that with arginine the "seedlings" exhibited a greater tendency to develop more than two "cotyledons", mostly three. At the lowest concentration, 0.00063 *M* of arginine nitrate, some striking individuals with exceptionally broad leaves were seen (Figure 10), similar to those found at 0.01 % of glycine. In these leaves several veins and some solitary stomata were found, whereas the ordinary neomorphs of *Oenanthe aquatica* do not possess stomata. Thus the broad leaves approach the normal ones, confirming the view that the neomorph leaves are in fact organs comparable to normal leaves.

L(+)-Alanine allowed the neomorphs to grow well even at as high a concentration as 0.01 *M* (pH 5.85), the favourable effect appearing within as little as 2 months, but further cultures would be required for more exact comparisons.

L(—)-Leucine, tested at 0.01 and 0.001 *M* (pH 5.7—5.1), proved much more inhibitory than glycine and arginine. Within half a year only minute nodules were formed, their growth and condition being better at the lower concentration.

DL-Alpha-aminobutyric acid was tested at 0.001 and 0.002 *M* (pH 5.7). The cultures were followed for 10 months, during which time the neomorphs produced a considerable number of minute embryonic individuals and small

coralloid aggregates, mostly yellowish or pale-green, partly brown. No definite seedling-like individuals were to be seen.

L(+)-Valine, tested at 0.01 and 0.001 *M* (pH 5.6—5.7), was even more inhibitory than leucine. At the higher concentration the inoculum died, while at the lower concentration, within 8 months, a thalloid aggregate was formed, about 1 cm. in diameter, partly brown, partly colourless.

e) *Growth of the Neomorphs with an Amine.* Since Virtanen and Linkola (1946, 1957) had shown with pea plants that amines may cause morphological changes similar to amino acids, an experiment was conducted with phenylethylamine, which had also been employed by these authors. In the presence of 0.001 *M* of this substance, added to the basal solution B with 10 g. of sucrose per litre (initial pH 5.9), the neomorphs grew fairly well, forming both large aggregates and small nodules, yellowish or pale-green in colour. Leaflike organs and long thin roots were also to be seen. No return to normal growth appeared within 10 months, after which time the culture, showing morbidity, was discontinued.

f) *Growth of the Neomorphs with Pyrimidine Derivatives.* Uracil and thymine were tested in view of their role as nucleic acid constituents. At 0.001 *M* both were able to keep up the neomorph pattern of growth during the culture period, which was with uracil 8 months, with thymine 6 months. With uracil growth was fairly good, with thymine much more slow. The neomorphs formed nodules and thalloid aggregates but no seedlinglike individuals. Thymine, in contrast to uracil, allowed the development of some long roots. Towards the end of the culture period the neomorphs turned dark brown.

g) *Growth of the Neomorphs with Ribose in the Absence of Amino Acid.* Ribose, as a constituent of ribonucleic acids, seemed interesting. D(—)-ribose was added to the basal solution B to give the concentration 0.0292 *M* (4.380 g. per litre), corresponding to 10 g. of sucrose per litre. This solution was applied partly as such, partly with 1, 2 or 4 g. of sucrose per litre, the initial pH varying between 5.9 and 6.3. Neomorphs from a culture with 0.004 *M* of arginine-monohydrochloride, in which no transition to normal growth had taken place within 10 months, served as starting material. The effect of ribose was surprising, as an inhibition of growth in size was accompanied by a transition to normal growth which took place more rapidly than when sugar was present in the form of sucrose alone. In the latter case the transition to normal growth has been found to require several months (cf. c), while with ribose it became apparent within 3—6 weeks (Figure 14—17). It is remarkable that development was best when a small amount of sucrose (1 g. per litre) had been added with the ribose, in which case the normal shoots were initiated within 3 weeks, while in the other cases the transition

was only apparent after 6 weeks. Moreover, the greater amounts of sucrose, though promoting the growth of the neomorphs, delayed transition, while ribose alone restricted both kinds of growth.

The time required for transition depends also on the light conditions. The above-described experiment was carried out during a light season (February to March). Another experiment of the same kind was carried out in a dark season (September to January) and with another starting material. With ribose+1 g. of sucrose per litre normal shoots were initiated within 3 months, while with 10 g. of sucrose alone the neomorph pattern of growth continued for 4 months at least.

Thus in the two experiments made at different seasons and with different starting material, ribose proved to promote the transition of the neomorphs to the normal pattern of growth.

h) *Morphology of the Neomorphs*. A fundamental difference between the neomorphs and normal plants is that the neomorphs are adapted to a submerged growth while the normal plants form aerial shoots.

The initial stages consist of undifferentiated nodules. When more advanced, these may assume a fusiform shape with a shoot end and a root end, and a central vascular strand may be seen. Sooner or later the shoot end manifests its character by dividing into two or more lobes (Figure 8). Initially the lobes may be roughly cylindrical with one vascular strand, but later on they grow into semicylindrical or more flattened leaves with several vascular strands (Figure 13). The general appearance of the neomorph leaves and their position in the seedling-like individuals corresponds to the cotyledons of the normal plant. The homology of the two is further supported by the fact that the neomorph stem has a central vascular strand like the normal hypocotyl (Figure 11). No stem will be formed after the leaves, but the number of leaves may increase and leaflike organs or new individuals may be formed from outgrowths at various points of the neomorphs (Figure 4, 9, 16). The root is thin and may possess a few branches, but often its development is more or less, if not entirely, suppressed (Figure 4). Both the outward appearance and the anatomical structure justify the view that the seedling-like neomorphs in fact correspond to cotyledon-stage seedlings, though various differences do exist. For instance, the neomorph leaves are much more succulent and more linear than the normal cotyledons (Figure 13, 19). On the whole it can be stated that the neomorphs of *Oenanthe aquatica* may reach the organization level of the cotyledon-stage seedlings but no further. On the other hand, depending on nutritional conditions, the neomorph may form thalloid individuals. These may be flat and leaflike, or present a coralloid appearance, and large aggregates may be formed by proliferation (Figure 3).

As a rule, the outgrowths are colourless even when developing from green parts in the sunshine. This is partly due to the fact that they arise from the epidermis, which is usually poor in chloroplasts; but a physiological suppression of chlorophyll formation must be involved, too, when it is accompanied by an inhibition of development. Thus, under unfavourable conditions, the grain-shaped nodules detached from the outgrowths may multiply, producing only colourless embryonic stages, i.e. minute polarly differentiated individuals, or mere nodules. Under favourable conditions the more advanced individuals assume a more or less green colour. Thalloid individuals are often partly green, partly colourless.

The adaptation to submerged growth is illustrated by the fact that stomata are lacking, with the exception of particular leaf types in which solitary stomata might be found; these leaves developed at a low concentration of amino acid and are in certain respects intermediary between neomorph and normal leaves (Figure 9, 10). The neomorph consists mainly of parenchyma and, at young stages, of meristem. The cells of the parenchyma enlarge considerably. A striking feature is that the intercellular spaces are filled with a substance stainable with ruthenium red and other dyes (Figure 11, 12). This indicates that either pectic substances or some similarly staining carbohydrates are present (cf. Henglein 1958). The cell walls, and to some extent the cell contents, also stain with ruthenium red. The cytoplasm stains reddish with pyronine (Unna's reagent), indicating the presence of ribonucleic acid. In the epidermis the cells are mostly smaller than in the parenchyma, chloroplasts are scarce or lacking, and the cytoplasm contains no coarse particles. The epidermis is readily converted into meristem. A peculiar feature is that it may sometimes show an ordinary intercellular space debouching direct into the nutrient medium. In the vascular strands the xylem is poorly developed, and a mechanical system is lacking. Besides the submerged growth, the heterotrophic nutrition is evidently responsible for many of these aberrant characters.

In this connection it may be pointed out that intercellular spaces filled with water appear in aseptic cultures of wheat roots, and that the gas in other parts of the roots consists of carbon dioxide (Burström 1959). It seems probable that the formation of carbon dioxide in wheat roots and the formation of carboxylated compounds in the intercellular spaces of the neomorphs are related phenomena.

Discussion

From the present work it appears that in *Oenanthe aquatica* the neomorph pattern of growth induced by glycine can be sustained by various other amino acids, by two pyrimidine derivatives, and by an amine. Although sustain-

ing power is no direct proof of inducing power, it shows that the substances concerned, though varying in effectiveness, restrict differentiation in a more or less similar manner. As in the case of the adaptation to glycine when neomorphosis is induced, the adaptation of the neomorphs to new amino acids or to other effective substances may require a long time during which the transferred individuals exhibit morbidity before new growth takes place. Thus the neomorphs are sensitive to changes in the nutrient medium.

Morphogenetic effects of amino acids have earlier been established in both higher and lower plants. Virtanen and Linkola (1946, 1957) found that pea plants assume a strange form, characterized by increased branching, reduced leaves and tufty roots, if nitrogen is supplied in excess as certain amino acids and amines which are tolerated only at low concentrations. A comparable phenomenon seems to be the "frenching" of tobacco, which has been dealt with in a series of studies by Steinberg and his collaborators (Steinberg 1947, 1949, 1952; Steinberg, Bowling and McMurtrey 1950). The symptoms of this disease include inhibition of stem elongation and a profuse development of narrow, even strap-shaped, leaves. They are accompanied by an accumulation of amino acids in the leaves and can be induced by amino acids in aseptic culture.

Studying the prothallia of a fern, Sossountzov (1954 and previous papers cited in this paper) found that their shape and growth are markedly affected by glycine and other amino acids.

In the above-mentioned examples of pea and tobacco the morphogenetic effects of amino acids do not exceed the range within which the characters of the species or of the genus are still recognizable, while in the present case, termed neomorphosis, the effects are so profound that even the family characters are hardly recognizable. Moreover, in the former case it is the organs of the original seedlings which appear modified, while in the present case the original seedlings undergo a long period of morbidity after which a new pattern of development initiates from the growing points. From these embryonic units become detached and multiply and may reach a differentiation level comparable to cotyledon-stage seedlings but adapted to submerged growth, all development stages being able to form new embryonic units from their outgrowths. Thus neomorphosis implies a cycle of development which is restricted to a more or less embryonic stage.

In this connexion it is of interest that Steward and his collaborators succeeded in deriving embryonic stages direct from vegetative cells which were disassociated from carrot roots by a very promising method (Steward and Pollard 1957, 1958; Steward, Mapes and Smith 1958). The embryonic stages could be reared to mature plants, but the cultures could also be continued at a cellular level. It may be pointed out that coconut milk was

added to the nutrient medium, and that considerable amounts of hydroxyproline were found in the tissue cultures of carrot and potato. As the authors say (Steward and Pollard 1958), the question arises how far the events of embryology in the ovule are determined by nutrition and how far they presuppose fertilization.

All the above-mentioned data are compatible with the view that amino acids are closely concerned with differentiation. The morphogenetic effectiveness of amino acids has proved so strong that it can well be compared with hormonal action; this Steinberg (1947, p. 88) has done, focussing attention on the role of the ordinary metabolic products in growth correlations. Evidently differentiation depends on changes in equilibria between the many substances involved in metabolism. Illustrative examples of the equilibria between growth substances have been cited by Skoog and Miller (1957). In the present case the morphogenetic significance of the equilibria between the major intermediary metabolites become more pronounced.

In these equilibria a competitive interaction seems to play an important role. This appears as a physiological antagonism between various amino acids, between various sugars, and between these two groups of substances. The antagonism between various sugars has recently been dealt with in detail by Stenlid (1959). In the present work it is represented by the morphogenetic antagonism between ribose and sucrose, ribose promoting the transition of the neomorphs to normal growth, in particular when a small proportion of the total sugars was present as sucrose. In spite of inhibiting growth in size, ribose thus promoted differentiation, which shows that growth in size and differentiation do not necessarily run parallel. The antagonism between amino acids and sugars appeared from the fact that in the absence of sucrose glycine proved much more toxic to the seedlings of *Oenanthe aquatica*.

Since the equilibria between intermediary metabolites have a morphogenetic significance it may reasonably be assumed that their antagonism is displayed at definite sites in the cellular structure. These sites may well reside in the cellular organelles, nucleus, plastids, mitochondria and microsomes. A tentative interpretation of the morphogenetic antagonism between amino acids and sugars can be based on the theory that nucleic acids are concerned with protein synthesis which, in turn, affects, and is affected by, carbohydrate synthesis. It may be remembered that in the neomorphs both kinds of syntheses seem to be affected (Miettinen and Waris 1958). The present results with ribose could be taken to mean that syntheses involving ribonucleic-acid-containing bodies are concerned. This focusses attention to the mitochondria and microsomes. In this connexion it is interesting that

Bonner has recently attributed a particular significance to the microsomes, for he suggests that differentiation may consist merely in enrichment or impoverishment of the cell in particular kinds of microsomes, which differ in their ribonucleic acids and synthesizing power; the microsomes, in turn, are supposed to receive their ribonucleic acids and proteins from the nucleus. In the case of mitochondria, it has been shown that they are subject to changes in number and in content of ribonucleic acids during metabolism and development (Lindblad 1959).

The present work offers some evidence for the hypothesis that various organs are differently affected by amino acids. Thus in *Oenanthe aquatica* there appears a difference in the nutritional requirements between hypocotyl and epicotyl, and between cotyledons and foliage leaves. On the other hand, the meristems exhibit a surprising degree of plasticity.

Summary

In four species of the family Umbelliferae a fundamental morphogenetic change, called neomorphosis, has been induced by critical concentrations of certain amino acids. The neomorphs of *Oenanthe aquatica*, reported previously in brief, have now been described in detail.

In *Oenanthe aquatica* the neomorph pattern of growth induced by glycine could be sustained by various other amino acids, by two pyrimidine derivatives, and by an amine. It consists in a vegetative cycle of reproduction in which development is restricted within an embryonic range, commencing from callus-like nodules and leading, at the most, to individuals corresponding to cotyledon-stage seedlings but adapted to submerged growth.

If amino acid is omitted but sucrose present, the neomorph pattern of growth may continue for several months, but eventually a transition to normal growth takes place by the formation of new growing points. Thus, in the species in question, neomorphosis does not involve any irreversible change of the genome.

The transition to normal growth is considerably accelerated by ribose, although ribose inhibits growth in size.

The results indicate that amino acids are closely concerned with differentiation.

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Some Effects of Stereoisomeric Tartrates on the Growth of *Lepidium* Roots

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Introduction

The specific effect of the different stereoisomeric tartrates upon root growth in *Lepidium sativum* L. was demonstrated in a previous paper (Aasheim 1958). It was shown that D(+)tartrate did not inhibit root elongation at any stage of growth. L(—)tartrate inhibited the elongation from about 40 hours and onwards. Mesotartrate also was inhibitory when the seedlings had grown for about 40 hours, and consistently more active than L(—)tartrate. DL tartrate was highly inhibitory at concentrations above 0.005 moles per litre.

The purpose of the work presented in this paper was to investigate further the inhibitory effects of tartrates in relation to other acids of related molecular structure (malic, succinic, fumaric, and malonic acids).

Materials and Methods

The dicarboxylic acids used in the present work were analytical grade chemicals from A/G Merck, Germany, and A/G Fluka, Switzerland. The technique of the germination test is described by Aasheim (1958). All the acids were neutralized with sodium hydroxide to a pH-value of 6.0—7.0, except those referred to in Table 5 where potassium hydroxide also was used. Except for the experiments reported in Tables 2 and 3, the seeds were of the harvest of 1956 and obtained from J. E. Ohl-sens Enke, Copenhagen. They were thus not of the same batch as the ones used in

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Table 1. *Combinations of the various acids (neutralized) as growth media for Lepidium seedlings.* The growth media are made up of 5 ml solution a plus 5 ml solution b. The results are expressed as length of roots and the germination percentage after 64 hours of germination. Each value is based on the average of at least 100 seeds. The experiments were carried out during September—December 1958, with seeds harvested in 1956. Root length mm., germination %.

Solution a	Solution b									
	5 ml 0.01 M D(+) tartrate	5 ml 0.01 M L(—) tartrate	5 ml 0.01 M meso- tartrate	5 ml 0.01 M D(+) malate	5 ml 0.01 M L(—) malate	5 ml 0.01 M succin- ate	5 ml 0.01 M fumar- ate	5 ml 0.01 M malon- ate	5 ml distil- led water	
	root length, germination	root length, germination	root length, germination	root length, germination	root length, germination	root length, germination	root length, germination	root length, germination	root length, germination	root length, germination
5 ml 0.01 M D(+)-tartrate	24.4 90	7.0 88	7.8 86	24.8 78	29.1 88	28.0 85	24.9 81	25.5 89	25.4 85	
5 ml 0.01 M L(—)-tartrate	16.2 83	8.4 82	21.6 81	21.7 83	22.0 84	19.7 87	16.9 83	21.0 77	
5 ml 0.01 M mesotartrate	7.7 79	8.9 80	8.4 85	8.7 68	8.1 83	8.8 78	8.8 77	
5 ml 0.01 M D(+)-malate	27.4 85	25.1 82	28.4 87	24.8 92	25.0 88	24.4 86	
5 ml 0.01 M L(—)-malate	23.8 77	23.3 77	24.1 86	21.6 84	25.5 87	
5 ml 0.01 M succinate	31.7 87	22.7 82	20.7 76	28.0 80	
5 ml 0.01 M fumarate	27.7 86	24.9 93	29.4 88	
5 ml 0.01 M malonate	18.6 93	25.5 90	
5 ml distilled water	26.1 86	

the previous study. The seeds referred to in Table 2 were obtained from A/S Norsk Frø, Norway, and were of the 1955 harvest. Those dealt with in Table 3 were obtained from J. E. Ohlsens Enke, and were harvested in 1957. All seeds were stored at laboratory temperature.

It is a well-known fact that the growth rate of seedlings is affected by the age of the seeds and the storing conditions. An endogenous annual rhythm may also be responsible in part for variations in the growth of roots.

All tables are therefore supplied with information about the dates on which the experiments were carried out. Comparison between experiments carried out at great intervals of time must be done with caution. For instance, the rates of root elongation listed in Table 5 (sodium tartrates) are much greater than for the corresponding treatments in Table 1. The only difference between the two sets of experiments is that the experiments in Table 5 date from April—May 1958, while those in Table 1 were carried out during September—December 1958.

The rate of root elongation in water was determined as a control simultaneously with the establishment of the effects of the various treatments. The growth of the controls was fairly constant, and the data contained in a given table, are therefore, considered comparable.

Experimental Results

Evenari *et al.* (Evenari 1949) have pointed out the synergistic effect of citric and malic acids upon seed germination. Given alone, the two acids

Table 2. *Inhibition of root elongation by L(—)tartrate and its partial reversion by malate. Duration of germination 64 hours. Experiments carried out 16.—19. January 1957 with seeds harvested in 1955.*

Growth medium		Average length of roots mm	Standard error (root length) $\sqrt{\frac{S(x-\bar{x})^2}{n(n-1)}}$	Germination %	Number of seeds
No. 1	Distilled water	39.6	1.59	94	50
No. 2	0.005 <i>M</i> D(+)tartrate ...	46.6	1.26	92	50
No. 3	0.005 <i>M</i> L(—)tartrate ...	32.9	1.08	93	75
No. 4	0.005 <i>M</i> L(—)tartrate ...	38.1	1.38	96	50
	0.005 <i>M</i> D(+)malate ...				
No. 5	0.005 <i>M</i> L(—)tartrate ...	41.3	1.19	84	75
	0.005 <i>M</i> L(—)malate ...				
No. 6	0.005 <i>M</i> L(—)malate ...	43.7	1.19	95	75
No. 7	0.005 <i>M</i> D(+)malate ...	46.2	1.40	93	75
No. 8	0.005 <i>M</i> D(+)tartrate ...	48.5	1.41	91	75
	0.005 <i>M</i> D(+)malate ...				
No. 9	0.005 <i>M</i> D(+)tartrate ...	42.8	2.10	96	50
	0.005 <i>M</i> L(—)malate ...				

did not inhibit germination, but when combined, they greatly suppressed germination. It might therefore be interesting to study the effect of the different stereoisomeric tartrates both alone and in combination with other dicarboxylic acids.

Table 1 presents the results of such experiments. As noted before (Aasheim 1958) the germination percentages are very little or not affected by the various treatments. On the other hand the lengths of roots exhibit a more complex pattern in response to the acids. It is to be noted that only germinating seeds were included in the calculation of average root lengths.

In Table 1 is shown the responses to the various acids at a concentration of 0.005 moles per litre (5 ml of 0.01 *M* acid plus 5 ml of distilled water make 10 ml of 0.005 *M* acid). The seeds were germinated for 64 hours. The inhibition caused by mesotartrate is noticeable. L(—)tartrate was slightly inhibitory. The inhibition caused by mesotartrate was not reversed by the addition of equal quantities of any of the other acids, whereas the slight inhibition by L(—)tartrate seems to be reduced by the addition of malates and succinate. This will be dealt with below.

The values in the diagonal from top left to bottom right (except the last one) give the results of germination in 0.01 *M* acid. Here the inhibition by L(—)tartrate is more pronounced.

Except in the case of D(+)tartrate plus L(—)tartrate (which together constitute 0.01 *M* DLtartrate) none of the combinations seem to act in a synergistic way. The effect of DL tartrate will be dealt with below.

Table 3. *Inhibition of L(—)tartrate and the effect of malates and succinate there on.* 64 hours of germination. Each value represents the average of 50 seeds. 9th of January 1959. Seeds harvested in 1957.

Growth medium		Average length of root mm	Standard error (root length)	Germination %
No. 1	Distilled water	40.1	1.33	100
No. 2	0.005 <i>M</i> D(+)tartrate	41.5	1.39	98
No. 3	0.005 <i>M</i> L(—)tartrate	28.9	1.61	96
No. 4	0.005 <i>M</i> L(—)tartrate	31.8	1.45	92
	0.005 <i>M</i> D(+)malate			
No. 5	0.005 <i>M</i> L(—)tartrate	34.7	1.67	96
	0.005 <i>M</i> L(—)malate			
No. 6	0.005 <i>M</i> L(—)tartrate	27.5	1.41	98
	0.005 <i>M</i> succinate			
No. 7	0.01 <i>M</i> D(+)malate	33.0	1.40	100
No. 8	0.01 <i>M</i> L(—)malate	36.4	1.83	96
No. 9	0.01 <i>M</i> succinate	33.3	2.08	98

When studied over a prolonged period of time, the inhibition caused by L(—)tartrate seems to be somewhat variable even within the same batch of seeds. Within some years, the seeds seem to lose their sensitivity to L(—)tartrate. The seeds referred to in Table 1 were not considered entirely suitable for the study of the effect of L(—)tartrate, which was, therefore, studied on two other batches of seed (Table 2 and Table 3).

In Table 2 the average length of roots after 64 hours in 0.005 *M* L(—)tartrate is significantly lower than in either pure water or 0.005 *M* D(+)tartrate. When equal quantities of malates were added, the inhibition was less. The difference in root length between medium No. 3 and No. 4 is statistically significant and of course also between No. 3 and No. 5. But it is impossible to say for sure from the results in Table 2 that L(—)malate is more effective in relieving the inhibition than is D(+)malate. (An ordinary statistical *t*-test gave a $t=1.75$.)

Table 3 shows a similar experiment carried out two years later. In this experiment there is also an obvious inhibition caused by 0.005 *M* L(—)tartrate. This inhibition appeared to become reduced when either of the malates was added (media No. 4 and No. 5), but the difference between medium No. 3 and No. 4 is not statistically significant ($t=1.37$ with 92 degrees of freedom). The difference between No. 3 and No. 5 is, however, statistically significant with a $t=2.51$, which gives a probability *P* between 0.02 and 0.01. Succinate could not reduce the inhibition caused by L(—)tartrate.

These experiments thus give the general impression that the inhibition caused by L(—)tartrate can be reduced by the addition of malate. L(—)malate seems to be more effective in this respect than D(+)malate, but the

Table 4. *Effect of addition of Ca- and Mg-ions to the various Na-tartrate media.* Duration of each experiment 64 hours. Concentrations in moles per litre of final growth medium. Each of the combinations represents the average of at least 50 seeds. Experiments carried out during October 1958.

Cation addition	Water		0.01 M D(+) tartrate		0.01 M L(—) tartrate		0.01 M DL tartrate		0.01 M mesotartrate	
	root length mm	germi- nation %	root length mm	germi- nation %	root length mm	germi- nation %	root length mm	germi- nation %	root length mm	germi- nation %
0	26.8	86	26.9	88	18.3	82	7.6	89	7.6	82
0.005 M CaSO ₄ . . .	32.4	82	30.9	92	22.4	74	16.6	90	10.7	88
0.005 M MgSO ₄ . . .	25.2	90	28.1	88	17.2	82	6.0	78	8.9	80
0.005 M CaSO ₄ . . . } 0.005 M MgSO ₄ . . . }	31.7	88	30.1	80	17.8	80	17.1	82	10.7	86

results obtained so far are not conclusive on this point. L(—)malate has the greatest resemblance to L(—)tartrate in steric configuration.

The heavy inhibition caused by 0.01 M DL tartrate (Table 1) is remarkable. DL tartaric acid is less soluble in water than any of the other isomers. Therefore it was first thought of a complex between DL tartaric acid and some metal as the inhibiting principle. DL tartaric acid might for instance act as a stronger chelating agent than any of the other isomers. An addition of microelements gave, however, no reversion of the inhibition. Two growth media were studied. Both contained sodium DL tartrate in a final concentration of 0.01 moles per litre. In addition, the first medium contained the following:

5.00 mg/l ferric citrate	0.22 mg/l zinc sulphate
2.86 „ boric acid	0.08 „ cupric sulphate
1.81 „ manganese chloride	0.02 „ molybdic acid

The concentrations are given as mg per litre of the final growth solution and are the same as those in the water culture solution of Hoagland and Arnon (1950). The second medium contained the same elements in a concentration ten times higher. None of these media gave significantly better elongation of the roots than did DL tartrate alone.

Very soon, however, calcium was found to act as an antagonist to DL tartrate as will be seen from Table 4. Calcium alone seems to enhance root elongation to some degree. The power of calcium to reverse the inhibition by DL tartrate is clearly seen: the average root length was 7.6 mm. in pure DL tartrate, 16.6 mm when calcium sulphate was added, and 17.1 mm when both calcium and magnesium were added.

The elongation in mesotartrate and L(—)tartrate was also stimulated by calcium, but not all to such an extent as in the case of DLtartrate, and not greater than in roots receiving no tartrate. Magnesium had very little or no effect on any of the plants growing in solutions of tartrates.

This effect of calcium upon the inhibition by DLtartrate can be explained by the fact that the calcium sulphate solution forms a precipitate with DL tartrate and not with any of the other tartrates. The precipitate was not detected until the experiments in Table 4 were finished, because the salts were added separately to the filter paper on which the seeds were to germinate.

In Beilsteins Handbuch der organischen Chemie, 4. Aufl., II. Ergänzungsw. B. 3, p. 322, 334, 336, 339, Springer Verlag, Berlin 1942, the solubility of the different stereoisomeric calcium tartrates are given thus:

100 g aqueous solution contains at

25°C	0.040 g	water-free calcium D(+)tartrate
20°C	0.025 g	hydrated calcium L(—)tartrate (4H ₂ O)
25°C	0.005 g	water-free calcium DLtartrate
20°C	0.032 g	hydrated calcium mesotartrate (3H ₂ O)

Now it is evident that DLtartrate could not act as an inhibitor in the presence of calcium because it was precipitated and could not enter the roots. But it is also highly probable that the inhibitory effect of DLtartrate in the absence of externally supplied calcium is due to a precipitation of metals, primarily calcium, inside the roots. Calcium is generally known to play an important, but still not wholly understood part in the growth of roots.

Coolil (1951) has studied the influence of various sodium and potassium salts upon the growth of *Avena* seedlings. He found that potassium was more effective in stimulating the growth of leaves than was sodium. But he was unable to demonstrate any difference between the two cations in regard to the growth of roots. It might be interesting to see if there was any difference between potassium and sodium tartrates in regard to the growth of *Lepidium* roots. Table 5 shows that such a difference does not exist except in the case of DLtartrate.

The roots grown for 64 hours in 0.01 M potassium DLtartrate reached an average length of 5.0 mm versus 7.4 mm in sodium DLtartrate. The average root length in a mixture of sodium- and potassium- DLtartrate was intermediary. The difference in root length between the roots grown in potassium DLtartrate and in sodium DLtartrate is highly significant. An ordinary statistical *t*-test gave a *t*=6.17 with 167 degrees of freedom.

It is impossible at present to see why potassium DLtartrate should inhibit more than sodium DL-tartrate.

Table 5. *Effect of potassium and sodium tartrates, and the mixture of potassium and sodium tartrate on roots of Lepidium.* Results are given as average root length (mm) and germination percentage after 64 hours of germination. Each value represents the average of 100 seeds. Experiments carried out during April—May 1958.

Anion	Potassium		Sodium		Potassium : sodium 1 : 1	
	Root length mm	Germ. %	Root length mm	Germ. %	Root length mm	Germ. %
0.005 <i>M</i> D(+)tartrate	37.3	76	36.0	85	—	—
0.005 <i>M</i> L(—)tartrate	25.0	79	25.6	86	—	—
0.01 <i>M</i> D(+)tartrate	32.4	85	30.9	91	32.6	84
0.01 <i>M</i> L(—)tartrate	17.1	78	16.5	69	16.5	73
0.01 <i>M</i> DLtartrate	5.0	88	7.4	81	6.3	76

Discussion

The experimental results presented here may be viewed in the light of what is known about the metabolism of organic acids, especially tartaric acids, in plants. Unfortunately the knowledge in this field is not very extensive. Recent reviews on the subject are given by Vickery and Palmer (1954) and Stafford (1957).

Earlier, tartaric acid, and primarily the naturally occurring D(+)tartaric acid, was thought to be oxidized by malic dehydrogenase. Recently, Stafford (1957) has demonstrated the presence in plants of an enzyme capable of oxidizing L(—)tartaric and mesotartaric acids. The oxidation product was presumed to be either the keto- or enol-form of dihydroxyfumarate. The pathway would thus be the same as the one postulated by Thunberg (1920) for the oxidation of L(—)-, DL-, and mesotartaric acids by preparations of frog muscle. A specific enzyme which can oxidize D(+)tartaric acid has not yet been demonstrated in plants.

As to the reported effects of tartrates upon the elongation of *Lepidium* roots, the mechanism of inhibition by DLTartrate is already mentioned. Its binding of calcium and other cations inside the roots seems very probable. The application of DLTartrate may perhaps be a useful means of reducing the internal calcium ion concentration in plants when this is desired for experimental purposes.

It is more difficult to suggest a mechanism of action for the inhibiting mesotartrate. Mesotartrate is shown by Quastel and Scholefield (1955) to act as a specific inhibitor of pyruvate oxidation in rat kidney cortex. This inhibition could be completely reversed by small amounts of DLmalate or citrate. The inhibition of root elongation by mesotartrate reported in the present paper could not be reversed by malates. But still it might be worth while

to study the oxygen uptake and pyruvate conversion in *Lepidium* roots to see if the same system is operating here. Mesotartrate could also exert its effect through the enzyme system of Stafford (1957) already mentioned. If the oxidation of mesotartrate by that system would lead to conditions unfavourable to the elongation of the roots of *Lepidium* seedlings, one might seek a possible explanation for the inhibition in that system. But this is by no means demonstrated. The system demonstrated by Stafford oxidizes both L(—)tartrate and mesotartrate. Both tartrates inhibit the early elongation of *Lepidium* roots, both their effects are not similar. L(—)tartrate-inhibition can be reversed by malates, but the inhibition brought about by mesotartrate cannot. Cress seeds retain their sensitivity to the latter for many years, while their sensitivity to L(—)tartrate shows a tendency to disappear after some time. The elongation tests, therefore, indicate that these two acids exert their effects on two different systems. L(—)tartrate could, for instance, act upon L(—)malic dehydrogenase. This is indicated by the reversion of inhibition by malates, and will be more probable if it can be definitely shown that L(—)malate is more effective in this respect than D(+)malate.

A further discussion of this subject should, however, be postponed until some data concerning the respiration and biochemical activities of the roots, both in the presence and absence of the various acids, have been provided.

Summary

1. The elongation of roots and the germination percentage of the seeds of *Lepidium sativum* L. have been studied in the presence of stereoisomeric sodium tartrates and sodium salts of some other dicarboxylic acids.

2. The inhibitory action of DL tartrate upon the elongation of roots is tentatively ascribed to the precipitation of calcium or other metals inside the roots.

3. The inhibition of root elongation brought about by L(—)tartrate can be partially reversed by the addition of malates. It is indicated that L(—)malate is more effective in this respect than D(+)malate.

4. Mesotartrate also inhibits the elongation of roots, but this inhibition is not reversed either by malates, fumarate, malonate, or succinate.

5. The effect of potassium tartrates versus sodium tartrates was studied. No difference was observed except for 0.01 M DLtartrate. Potassium DLtartrate was more inhibitory than the corresponding sodium salt.

6. The mechanisms of action of these inhibitory tartrates are tentatively discussed.

The writer wishes to thank professor, dr. Poul Larsen for his great interest in this work and his valuable help.

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The Inhibition of Transport of Indoleacetic Acid by Phenoxyacetic Acids

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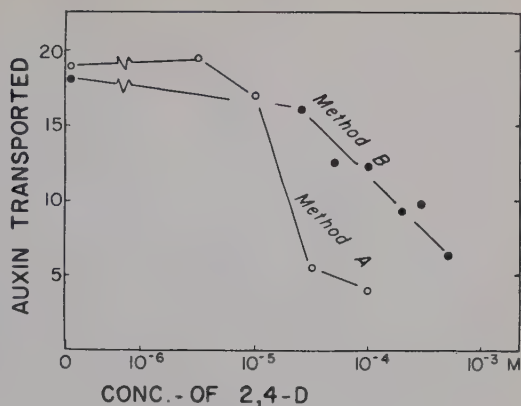
Two general types of inhibitors of transport of indoleacetic acid (IAA) are known: respiratory poisons and auxin homologues. The former have been utilized in studies of the role of metabolism in transport (du Buy and Olson 1940, Niedergang-Kamien and Leopold 1957). Some auxins and auxin homologues have been reported as inhibiting the transport of IAA (Niedergang 1954, Zwar and Rijven 1956, Hay 1956, Keitt and Skoog 1959). From these studies, then, a picture emerges of an auxin transport system which is driven by a metabolic system, which is sensitive to small changes in the molecule being transported, and which is obstructed by some substances with structural similarities to IAA. Such a picture of auxin transport is made tenuous, however, by the fact that studies of transport inhibition by auxin homologues could have been confounded by inhibitions of the uptake of the IAA and also by interference with the bioassay for the transported hormone.

The present study provides evidence that the inhibition of IAA transport by 2,4-D is a quantitative and specific inhibition of transport itself. The molecular structure essential for transport inhibition is then examined in an effort to establish what the nature of the inhibition might be.

Methods

The transport of indoleacetic acid (IAA) through sunflower stem sections was studied using hypocotyl sections taken from sunflower plants at an age of 2—3 weeks when the first pair of leaves had expanded to about $\frac{3}{4}$ of their final size.

Figure 1. *The quantitative inhibition of IAA transport in sunflower stem sections by 2,4-D. Method A: 2,4-D applied through the transpiration stream for 16 hours; Method B: 2,4-D applied in agar at stem section base for 30 minutes. Degrees curvature in the Avena test.*



In the first experiment reported, the sunflower stems were severed 2—3 cm. below the cotyledons and the shoots placed for 16 hours with their lower portion in 50 ml. of 2,4-D solution. The solution was thus taken up in the transpiration stream. Then the tissue which had been immersed in the solution was discarded and 5 mm. sections were cut from the first internode above the cotyledons. This will be referred to as method A.

In all subsequent experiments, 5 mm. sections were taken from freshly cut sunflower stems and treated by placing them, basal end down, on agar blocks containing the phenoxy acid (Method B). IAA (10^{-5} M) was applied with an Agla micro-syringe as a 2 μ l droplet to the apical end within a minute of the time when the basal end of the section was placed in contact with the 2,4-D. After 30 minutes, the sections were removed from the agar treatment block, blotted, and 6 sections placed on a fresh blank agar block ($9 \times 12 \times 1.2$ mm.). After a further interval of 90 minutes, the IAA which had been transported into the blocks was determined by the Avena curvature test. Each test reported represents the mean of twelve individual curvatures.

Results

The Quantitative Nature of the Inhibition

In the first efforts to quantitatively measure the effect of 2,4-D on IAA transport, the 2,4-D was introduced into the transpiration stream at concentrations from 3×10^{-6} M to 10^{-4} M. Epinastic responses in the shoots occurred at each concentration tested. The subsequent transport of IAA in the hypocotyls was markedly depressed by the 2,4-D as can be seen in Figure 1 (Method A.). The inhibition increased with increasing concentration, reaching 79 % at the highest treatment concentration, 10^{-4} M 2,4-D.

The technique used in this type of experiment is subject to a serious reservation, for it has been shown that 2,4-D can inhibit the uptake of IAA (Reinhold 1954). Since with this method of treatment 2,4-D will be present at the

Table 1. *Effect of removal of 1 mm. from the basal end of stem sections on IAA transport inhibition with 2,4-D (5×10^{-4} M).*

Exp.	Treatment	IAA transported (degrees curvature)	
		Sections intact	1 mm. removed after 30 min.
I	H ₂ O	13,9	9,5
	2,4-D	4,7	7,6
	Inhibition:	66 %	20 %
II	H ₂ O	20,4	15,8
	2,4-D	7,3	13,8
	Inhibition:	64 %	13 %

cut surface receiving the IAA, the effects on uptake and on transport cannot be distinguished.

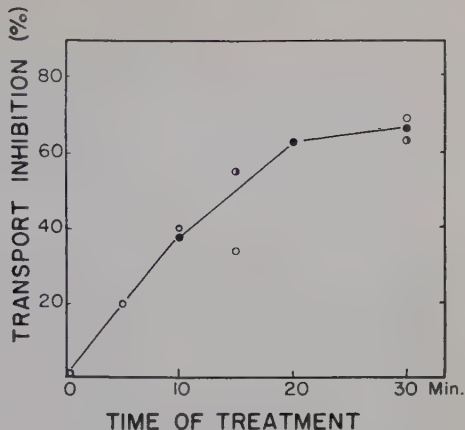
An experimental device was therefore introduced by which the 2,4-D treatment would not affect the IAA uptake. The technique used in all the subsequent experiments incorporates the essential feature that the 2,4-D and the IAA be simultaneously applied to opposite ends of the hypocotyl section. Consequently the 2,4-D and IAA must come face to face, so to speak, in the section itself and only there can the 2,4-D exert its effect on transport.

It was first established that the effects of 2,4-D presented at the base of the stem section were not due to an inhibition of IAA uptake at the apical end. This was shown by experiments in which the basal one mm. of tissue was cut from the base of each section after the 30 minute treatment with 2,4-D. It was found that most of the inhibition was removed by this operation (Table 1). The inhibitions were reduced from 66 % to 20 % in one experiment and from 64 % to 13 % in the other. We could conclude that the 2,4-D apparently was localized in the lower portions of the sections and would not affect IAA uptake at the apical ends during these short term experiments. The lower values for IAA transported through the control section which had the one mm. of tissue removed may be attributed to the loss of the IAA transported into that tissue during the 30 minute treatment interval.

Inhibition of IAA transport by basal application of 2,4-D was also found to be a quantitative effect. In Figure 1 (Method B) it can be seen that the inhibition of transport was proportional to the 2,4-D concentration and reached a level as high as 65 % at 5×10^{-4} M.

The extent of inhibition was proportional to the time of contact of the sections with the 2,4-D agar blocks (Figure 2). At 30 minutes contact time, inhibitions of transport reached a level of about 70 %.

Figure 2. The inhibition of IAA transport is proportional to the duration of treatment with 2,4-D (5×10^{-4} M). Three separate experiments indicated by the different symbols.



There are two additional factors which could be involved in the tests. One is the possible destruction of IAA at the cut tissue surfaces, and the second is the possibility of leakage of the 2,4-D into the agar blocks which are subsequently assayed in the Avena test. If the 2,4-D inhibits the Avena test one could confuse such an inhibition with a reduction of IAA transport. These factors were analyzed by placing 2,4-D treated tissue on agar blocks containing known quantities of IAA. The curvatures subsequently given by these blocks in the Avena test were compared with the activity of IAA-containing blocks which had been in contact with control tissue, and with blocks containing the same quantity of IAA but which had not been in contact with any tissue (Table 2). It can be seen that the 2,4-D treated tissue reduced the Avena assay result from 14.5° to 10.0° , a decrease of 4.5° . This decrease, which we will call the correction value, is not sufficient to account for the inhibition of 10.0° in the transport test. In 8 tests with 2,4-D, transport was inhibited an average of 7.9 degrees., while the correction values averaged 5.4 degrees.

Table 2. Interference with the Avena test by stem tissue treated with 2,4-D (5×10^{-4} M).

Treatment	Avena Curvature (degrees)		
	No tissue	H ₂ O treated tissue	2,4-D treated tissue
Agar containing 2×10^{-7} M IAA	12.3	14.5	10.0
Receptor block after IAA transport	—	18.5	8.5

Table 3. *The inhibition of IAA transport in sunflower stems by various chlorinated phenoxyacetic acids. Each acid applied as 5×10^{-4} M in agar at section base for 30 min.*

Inhibitor	No. of Expts.	IAA transport (degrees)		Per Cent Inhibited
		Control	Treated	
Phenoxyacetic acid	4	15.5	13.2	15
2-Cl	4	17.3	14.2	18
2,6-D	7	17.7	12.2	31
2,5-D	1	17.9	9.6	46
2,4-D	17	17.7	8.6	51
2,3,6-T	2	15.6	9.8	37
2,4,6-T	6	18.4	7.4	60
2,4,5-T	7	15.6	7.1	55
2,3,4-T	2	15.6	5.8	63
2,3,5-T	2	15.6	4.4	72
2,3,4,6-Te	1	17.7	11.5	35
2,3,4,5,6-Pe	1	17.7	11.1	37

Molecular Structure and Transport Inhibition

Having the assurance that 2,4-D actually inhibits the transport of IAA, we are prepared to examine the structural requirements for the action of the inhibitor. A series of phenoxyacetic acids was tested for activity in this regard, by the method utilized in the previous section — applying the phenoxy acid (5×10^{-4} M) at the basal end of the sunflower stem section for 30 min. while the IAA was applied at the apical end. The section was then blotted and transferred to a plain agar block for 90 minutes, and the IAA subsequently assayed in that receptor block.

A comparison of 12 phenoxyacetic acids was made, each with a different chlorination in the phenyl ring. The data for these experiments are summarized in Table 3. For each compound the transport through the control or untreated stem sections is reported, and the transport through the sections treated with the phenoxy acid. To facilitate comparison, the per cent inhibition for each compound is given in the last column.

Examination of Table 3 indicates that the effectiveness of the phenoxyacetic acids as inhibitors is strongly altered by the chlorination of the phenyl ring. The unchlorinated phenoxyacetic acid and the monochlorinated one were the least effective in inhibiting IAA transport. The dichlorinated group was markedly more inhibitory, and except for the 2,3,6-T, the trichlorinated acids were the most effective inhibitors found, ranging from 55 to 72 per cent inhibition. Further increases in chlorination did not appear to increase transport inhibition, though the number of experiments available on this point is limited.

Table 4. *Inhibitions of IAA transport by various phenoxyacetic acids (5×10^{-4} M) as corrected for interference in the Avena test.*

Inhibitor	No. of Expts.	IAA transport (degrees)		Correction (degrees)	Per Cent inhibited (corrected)
		Controls	Treated		
Phenoxyacetic acid	2	15.9	16.3	0	0
2-Cl	2	19.6	18.1	4.5	0
2,6-D	4	19.4	14.3	3.9	6
2,4-D	8	19.2	11.3	5.4	13
2,3,6-T	2	15.6	9.8	0	37
2,4,6-T	3	18.7	7.9	5.7	27
2,4,5-T	2	15.9	10.5	0	34
2,3,4-T	2	15.6	5.8	5.5	28
2,3,5-T	2	15.6	4.4	3.4	50
2,3,4,6-Te	1	17.7	11.5	0	35
2,3,4,5,6-Pe	1	17.7	11.1	1.7	28

Statistical analysis of these data shows that all of the inhibitions by the di- and tri-chlorinated acids are reliable at the 1 per cent level of dependability. The 2,6-D is significantly less inhibitory than the other dichlorinated acids tested, and the 2,3,6-T is also less inhibitory than the other trichlorinated acids tested in simultaneous experiments.

While these data do provide tentative evidence of the effects of chlorination on the effectiveness of phenoxy acids as transport inhibitors, their dependability is limited by the possible interference these compounds may exert on the Avena curvature test. In order to correct for any such interference, each of the phenoxyacetic acids was reexamined establishing a correction value for its possible interference in the Avena test. Each of the phenoxy acids was applied to transporting stem sections in the usual way, as well as to stem sections which were not transporting IAA, and the latter were then placed on agar blocks containing 2×10^{-7} M IAA. In this way, a correction value was determined for each of the phenoxy acids.

Transport tests for eleven phenoxyacetic acids and simultaneously determined correction values for each are reported in Table 4. If the correction value is subtracted from the apparent transport inhibition for each acid, a corrected inhibition value is obtained which we have expressed as per cent of the normal transport. Examination of these corrected inhibition values confirms the relative effectiveness of the unchlorinated, mono-, di- and tri-chlorinated acids. The di- and tri-chlorinations strongly increase the inhibition of IAA transport. Again the tetra- and penta-chlorinated acids are not more inhibitory than the tri-chlorinated ones, and may even be less inhibitory

though the number of experiments with these higher acids is limited. In these corrected tests, the phenoxyacetic acid and the mono-chlorinated one do not appear to have an inhibitory effect on transport.

Discussion

The experiments reported here provide evidence that the inhibition of IAA transport by 2,4-D is a real effect on transport itself, and that changes in the chlorination of the phenyl ring can greatly modify the effectiveness of the acids as transport inhibitors.

The analysis of chemical modifications of IAA transport took on a new requirement with the findings of Reinhold (1954) and of Johnson and Bonner (1956) that IAA uptake by tissues could be inhibited either by respiratory poisons or by 2,4-D. It has become evident that apparent inhibitors of IAA transport could actually be interfering with several aspects of the experimental process. These include (a) effects on the uptake of the IAA by the stem piece, (b) effects on the transport of IAA through the piece, or (c) effects on the bioassay utilized to detect the transported IAA.

With respect to the first possibility, a technique was developed by Niedergang-Kamien and Leopold (1957) to eliminate effects on uptake during transport tests with metabolic inhibitors. The same technique has been applied in the present experiments. By presenting the inhibitor at the base and the auxin at the apex the possible interference with uptake is minimized. The data in Table 1 establish that this dual application at opposite ends of the stem piece results in an inhibition of apparent transport which is markedly alleviated when the basal 1 mm of the stem piece is removed. Thus the bulk of the effective inhibitor must be in the basal end of the stem section, and must affect transport there, not at the apical cut surface where uptake of the IAA occurs.

The last possibility — that the apparent inhibition may be due to interference with the bioassay — has been met by the use of correction values for such interference by each acid tested.

Zwar and Rijven (1956) deduced from their experiments that 2,4-D and some related compounds (2,4-D-propionic acid, 2,4-D-butyric acid and 2,4,6-trichlorophenoxyacetic acid) inhibit IAA transport. Their experiments were carried out by applications of the suspected inhibitor in an agar block at the apical end of the stem section before the IAA was supplied for transport. While this device reduces the probability of interference with the bioassay, the apparent inhibitions could also include an interference with the uptake of IAA by the transporting tissue.

Hay (1956) examined the inhibition of IAA transport by 2,4-D and triiodobenzoic acid, and in these experiments he felt that there was not an appreciable interference by 2,4-D in the *Avena* test. Our conditions show a much more serious interference and demand the establishment of correction values. Hay also applied the inhibitors as mixtures with IAA at the apical end of the transport section. The inhibition which he observed could therefore be confounded by both uptake and bioassay interferences.

Morgan and Söding (1958) have suggested that naphthylphthalamic acid is also an inhibitor of IAA transport. Their tests were done by mixing the two chemicals in the donor block for the *Avena* test, and so their inhibitions may well include interference with IAA uptake.

Keitt and Skoog (1959) deduce from experiments on callus formation in tobacco stem explants that three benzoic acid derivatives may inhibit IAA transport: 2,3,6-trichlorobenzoic acid, 2,6-dichlorobenzoic acid and 2,5-dibromobenzoic acid. Since some benzoic acids do and others do not appear to inhibit transport, they suggest that there may be some stereochemical specificity for the inhibition.

The concept of specificity for transport inhibitors is borne out in the present study. Among the chlorinated phenoxyacetic acids there are enormous differences in inhibition effects.

Some deductions concerning the polar transport of IAA can be made on the basis of the facts at hand. There is evidence that small changes in the auxin molecule can strongly alter its ability to be transported in the *Avena* curvature test (Thimann 1935), and small changes in auxin analogues can enormously alter their inhibition effects upon transport. Two ways by which the transport of IAA can be visualized may be (a) as some polar field drawing the IAA along from cell to cell, or (b) as a consequence of some physical or chemical attachment of the IAA to another entity which may be either a polar material itself or a surface with polar qualities. Our knowledge of the molecular requirements for polar transport do not permit conclusions concerning these possibilities, nor do the facts concerning the inhibition of transport by metabolic inhibitors. However, the strong inhibitions of transport by chlorinated phenoxyacetic acids, and the marked influence of molecular structure on the effectiveness of these inhibitors is clearly more consonant with the second or attachment possibility than with the polar field possibility. Small changes either in the structure of the transported molecule or of the phenoxyacetic inhibitor can be logically interpreted as altering the ability of the IAA to be attached to a transport site.

If IAA is attached to a transport site, this could be either as a chemical bonding or as a physical adsorption. Examination of the effects of chlorination on the effectiveness of phenoxyacetic acids as inhibitors shows a striking

correlation with their effectiveness as adsorbants on charcoal (Leopold *et al.* 1960). Increases in chlorination are associated with increase both in transport inhibition and in adsorption, with the trichlorinated acids being most effective for both. The interesting possibility exists, then, that IAA may be adsorbed onto a surface or carrier for transport as has been suggested for transport mechanisms in general by Danielli (1954). The effects of auxin homologues as transport inhibitors might then be due to their being adsorbed onto these positions and hence interfering with the attachment of the IAA. The possibility of a chemical attachment of IAA in transport is also a good one, however, for just as the increases in chlorination enhance the adsorption qualities of the phenoxyacetic acids, there is evidence that they increase the pK values of the carboxyl (van Overbeek *et al.* 1951) or other dissociable group (Williams 1959). Therefore one would expect increased reactivity of the carboxyl groups to be associated with the molecular changes which we know to increase transport inhibition.

In none of the experiments with auxin homologues has the possibility been eliminated that transport inhibitors might be acting as metabolic inhibitors. It is very clear that IAA transport is dependent upon a metabolic driving force, and interferences with metabolism can sharply curtail transport (Gregory and Hancock 1955, Niedergang-Kamien and Leopold 1957). It is by no means certain, though, that measurements of metabolic rates with the application of transport inhibitors would establish whether or not the inhibitions were due to metabolic interference. It has been our experience that transport can be markedly inhibited by concentrations of metabolic inhibitors which produced no detectable effect upon metabolic rates (Niedergang-Kamien and Leopold 1957).

In conclusion, the factors influencing IAA transport can be interpreted as functioning through some attachment scheme. If IAA is attached to some site for transport, then changes in the IAA molecule itself which suppress this attachment will also suppress transport. Furthermore, auxin analogues may occupy the positions which IAA would otherwise fill, and in this way they may inhibit transport.

Summary

The transport of indoleacetic acid through sunflower stem sections has been studied using transport inhibitors of the phenoxyacetic acid series. Evidence is provided to establish that the previously reported inhibition of transport by 2,4-D is a real effect on transport itself. Then comparisons are made of the transport inhibition by various other members of the phenoxy-

acetic acid series. Eleven different chlorination substitutions are compared, and it is found that increasing inhibitions of IAA transport are obtained with increasing numbers of chlorine substitutions in the phenoxy ring. Maximal inhibitions are obtained with the trichlorinated phenoxyacetic acids.

The similarity of the chlorination effects on IAA transport inhibition and on adsorption onto charcoal are pointed out, and it is suggested that the transport inhibition effects may be related to adsorptive or chemical interference at some transport site of attachment.

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Experimental Separation of Gibberellin and Auxin Actions in Etiolated Pea Epicotyl Sections

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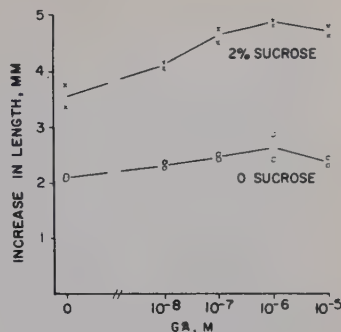
Introduction

Interactions between gibberellins and auxins have attracted much interest, and at least two related generalizations about them occur in the recent literature (see review by Brian 1959). The first is that the growth-promoting activity of gibberellin is dependent upon the presence of auxin. The second is the hypothesis that gibberellin acts through an auxin-mediated mechanism; that is, that gibberellin owes its action to a primary effect upon the auxin relations of the tissue. The results presented below do not agree with the latter hypothesis. They suggest, on the contrary, that the primary actions of gibberellin and of auxin, whatever each may be, are not closely connected, a view which is strengthened by a critical consideration of the literature.

Materials and Methods

Seeds of *Pisum sativum* L., var. Alaska (obtained from Asgrow, Inc., of New Haven, Connecticut) were soaked in tap water for 3—4 hours and then sown in thoroughly washed vermiculite (Mica-Gro Type B, supplied by Platt Seed Co., Branford, Connecticut). The seedlings were allowed to develop in a curtained cabinet in a dark room maintained at $30 \pm 1^\circ\text{C}$. They were exposed to dim green light at the time of harvest and, briefly, when the curtains were opened during normal activity in the dark room. The light source was a 15-watt Sylvania green fluorescent tube wrapped with at least 3 layers of dark green and 3 layers of amber DuPont cellophane, and was located about 30 cm. from the working area.

Figure 1. *Effect of 2 per cent sucrose on elongation response of S1 sections to GA. Control medium: 0.02 M phosphate buffer (pH 6.1). Each point represents the average of a lot of 10 sections.*



All sections were taken from plants having recurved apical crooks and third internodes between 15 and 50 mm. in length, a stage of development reached 7 or 8 days after planting. Except as otherwise indicated, 5-mm. sections taken 1 mm. below the apical crook were used; these are called "S1" sections (Purves and Hillman, 1958). In one series of experiments, "S5" sections were used; these had the same initial length but were taken 5 mm. below the apical crook. The sections were randomized in the basal medium, which was 0.02 M KH_2PO_4 — Na_2HPO_4 buffer, pH 6.1; and lots of 10 sections were then floated on 5 ml. of various media in 5-cm. petri dishes. Replicate lots were used in all experiments, so that each experimental treatment contained 2 lots of 10 sections. After approximately 20 hours of growth in darkness, sections were measured to the nearest 0.1 mm. by means of a dissecting microscope with an ocular micrometer.

In a series of experiments on the effects of gibberellic acid transported through epicotyl tissue, the methods of Galston and Warburg (1959) were employed. Seedlings were selected as described above, and apical segments of varying lengths were then harvested and decapitated. Lots of 10 segments were placed in beakers with the basal ends immersed in incubation media containing phosphate buffer and 2 % sucrose. The incubation time was 15 minutes except in the time-course study. S1 sections were then cut from these segments and grown in the usual manner. In these experiments, three replicate lots of 10 sections were used in each treatment.

As stated above, these experiments were done at a temperature of $30 \pm 1^\circ\text{C}$. This was done in order that plants from the present seed lot might reach the proper developmental stage within 7 days after planting. However, all of the data described below, with the exception of those of Figures 7 and 8, are confirmations of results obtained in the previous year with a different seed lot and at a temperature of $27 \pm 1^\circ\text{C}$.

Results

Responses of S1 Sections to GA, IAA, and Sucrose

The elongation of S1 sections is promoted by gibberellic acid (GA), indoleacetic acid (IAA), and sucrose. This observation confirms the report by Purves and Hillman (1958) and is also in agreement with earlier studies with

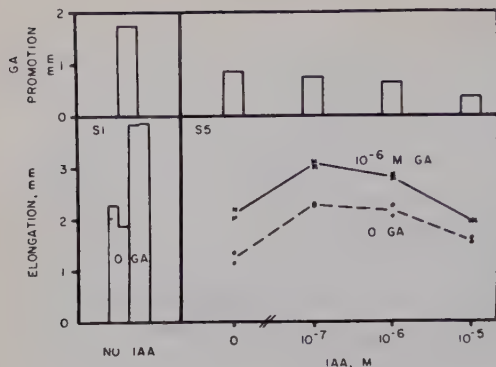


Figure 2. Effect of distance from apex on elongation response of etiolated pea epicotyl sections to GA. Lower histogram: response of S1 sections to 10^{-6} M GA in the absence of IAA. Curves: response of S5 sections to GA in the presence of various IAA concentrations. Upper histograms: magnitude of GA responses (GA-treated values minus control values). Control medium: 0.02 M phosphate buffer (pH 6.1) plus 2 per cent sucrose. Each point, and divisions of bars, represent replicate lots of 10 sections.

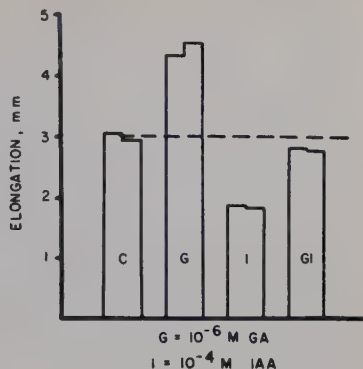
other types of pea sections (GA: Hayashi and Murakami 1953, IAA: Thimann and Schneider 1939, sucrose: Galston and Hand 1949).

The interaction between GA and sucrose in the growth of S1 sections is shown in Figure 1. In the absence of exogenously supplied sucrose, the growth promotion by GA is small or absent. However, in the presence of 2 % sucrose GA has a strong promotive effect; a synergism between sucrose and GA is observed. For this reason, the basal medium in most of the subsequent experiments included 2 % sucrose. There is no well-defined GA optimum, concentrations between 10^{-7} and 10^{-5} M having roughly the same activity. This is quite different from the complex interaction between IAA and sucrose in S1 growth described by Purves (1959), in which certain concentrations ($> 10^{-6}$ M) of IAA promote growth in the absence of sucrose but are inhibitory in its presence.

"Endogenous" and GA-Induced Growth

The magnitude of the GA-induced growth of sections is correlated with the "endogenous" (control) growth rate, and it has been suggested (Brian 1959) that this correlation may be explained in terms of higher residual auxin levels in those sections with higher endogenous growth. The data of Figure 2 bear on this hypothesis. S1 (apical) sections have a higher control growth and GA response than do the more basal S5 sections, but S5 sections treated with 10^{-7} M IAA grow as much as the S1 controls. However, the GA response of S5 sections so treated is still much lower than that of the S1 sections. Similar data have also been obtained in a comparison of the growth responses of S4 and S7 sections, and Purves and Hillman (1958) reported the same effect comparing S1 and S4 sections.

Figure 3. *Effect of inhibitory levels of IAA on response of S1 sections to GA.* Control medium: 0.02 M phosphate buffer (pH 6.1) plus 2 per cent sucrose. Divisions of bars represent replicate lots of 10 sections.



“Auxin-Sparing” by GA

If gibberellin promotes growth by virtue of an auxin-protecting (“auxin-sparing”) action, GA supplied to sections in the presence of supraoptimal IAA levels should inhibit growth. That it does not is shown in Figure 3. In the presence of 2 % sucrose, high concentrations of IAA are inhibitory to the growth of S1 sections (Purves and Hillman 1958); however, GA promotes growth even in the presence of inhibitory IAA concentrations. GA promotes elongation at all IAA concentrations tested, whether promotive, inhibitory, or ineffective; but the gibberellin-induced growth increment is frequently smaller at high IAA levels.

Other Mechanisms of Auxin-Mediated GA Action

Although the data of Figure 3 are prejudicial to the hypothesis that gibberellin acts through an auxin-sparing system, they are not opposed to other auxin-mediated mechanisms of gibberellin action. The following experiments,

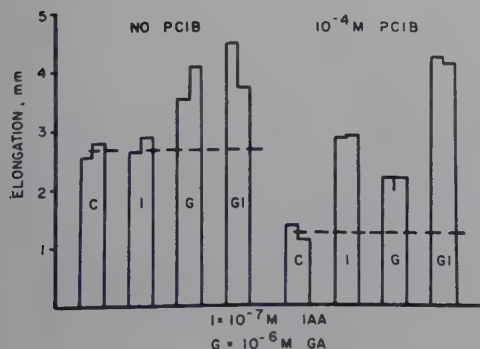


Figure 4. *Effects of PCIB on responses of S1 sections to IAA and GA.* Control medium: 0.02 M phosphate buffer (pH 6.1) plus 2 per cent sucrose. Divisions of bars represent replicate lots of 10 sections.

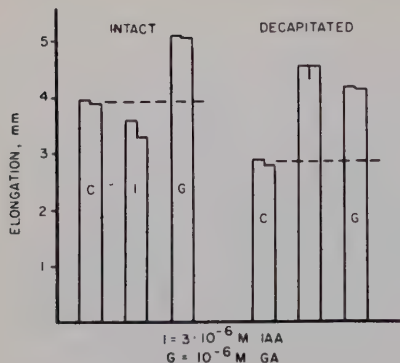


Figure 5. Effects of decapitation of test plants of responses of S1 sections to IAA and GA. Control medium: 0.02 M phosphate buffer (pH 6.1) plus 2 per cent sucrose. Divisions of bars represent replicate lots of 10 sections. (5th bar from the left should be marked I.)

however, which show that treatments strongly affecting the IAA responses of sections are without effect on the GA responses, are difficult to reconcile with any mechanism involving an intimate relationship between auxin and gibberellin actions.

Figure 4 shows the effects of α -(*p*-chlorophenoxy)-isobutyric acid (PCIB), which has been described as an antiauxin by Burström (1950). PCIB decreased the control growth of S1 sections and caused a marked change in the IAA-induced growth increment. On the other hand, PCIB induced no change in the GA response of the sections. No synergism between GA and IAA was found in either the presence or the absence of PCIB.

Similar effects were induced by decapitation of test seedlings prior to the excision of sections (Figure 5). Apical buds of seedlings were removed, and S1 sections were harvested 6 hours later. This treatment caused a reduction in the control growth rate and a profound change in the response to IAA, but the GA response was not affected. In other experiments it was shown that similar effects could be elicited by decapitation two hours before the experiment.

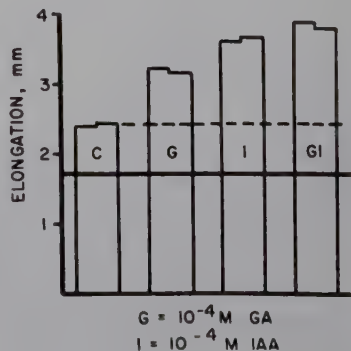
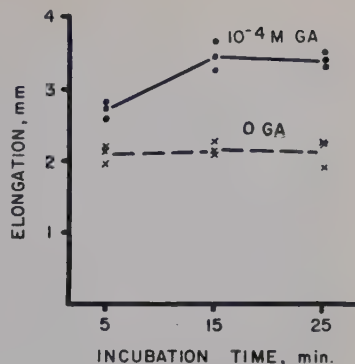


Figure 6. Effects of GA and IAA on elongation of starved S1 sections. Sections starved 24 hours in 0.02 M phosphate buffer (pH 6.1), then placed in media containing phosphate buffer plus 2 per cent sucrose for 20 hours. Solid horizontal line: growth during starvation period. Divisions of bars represent replicate lots of 10 sections.

Figure 7. Effect of time of incubation of segments on response of S1 sections to GA supplied basally to 10-cm. epicotyl segments. Control media: for incubation of segments, 0.02 M phosphate buffer (pH 6.1) plus 2 per cent sucrose; for growth of sections excised from segments, phosphate buffer plus 2 per cent sucrose plus 10^{-6} M IAA. Each point represents the average of a lot of 10.



Absence of GA-IAA Synergisms in S1 Sections

In some systems GA and IAA are synergistic in the promotion of growth. This is not true in etiolated pea epicotyl sections. When GA and IAA are supplied simultaneously, the growth responses are partially additive, although full additivity is seldom observed (Purves and Hillman 1958). Two exceptions to this rule have been reported, so the following experiments were undertaken.

Hayashi and Murakami (1953, 1958 b) found that "starved" sections responded to GA only in the presence of IAA or tryptophan, but we have been unable to repeat this observation. The responses of "starved" S1 sections to GA and IAA are shown in Figure 6. Sections were allowed to grow for 24 hours in buffer alone and then were transferred to media containing 2 % sucrose and GA or IAA. The growth of these sections was promoted by either GA or IAA alone, and no synergism between the two was observed. Similar results have been obtained with other concentrations of GA and IAA and with different periods of starvation.

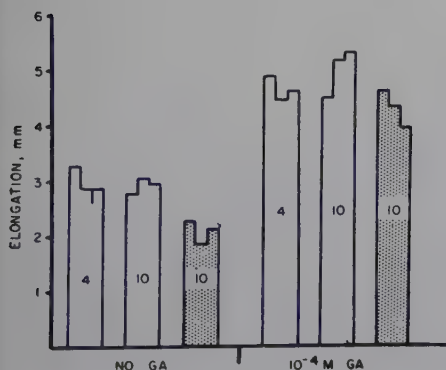


Figure 8. Effect of length of segment on response of S1 sections to GA supplied basally to epicotyl segments. 4: 4-cm. segment; 10: 10-cm. segment. Open bars: 10^{-6} M IAA given to sections excised from segments; shaded bars: no IAA. Control medium for incubation of segments and growth of sections: 0.02 M phosphate buffer (pH 6.1) plus 2 per cent sucrose. Divisions of bars represent replicate lots of 10.

Galston and Warburg (1959) have reported that GA and IAA are synergistic in promoting the growth of S1 sections when the GA is supplied to the base of a long epicotyl segment and IAA is supplied in the growth medium for a section subsequently excised from the segment. The data of Figure 7 confirm certain of the findings of Galston and Warburg. GA supplied basally to 10-cm.-long segments is active in promoting the growth of S1 sections excised from the segments, and the time course of GA pretreatment is in agreement with that reported by Galston and Warburg. On the other hand, these experiments failed to show a synergism between GA and IAA in this system (Figure 8); also, there was no effect of segment length on the magnitude of the GA response. These results are in disagreement with those of Galston and Warburg.

Discussion

"Endogenous" and Gibberellin-Induced Growth

In tissue sections and in decapitated seedlings it has been shown that greater responses to gibberellin are associated with greater endogenous growth rates (Purves and Hillman 1958, Radley 1958, Vlitos and Meudt 1957). Brian (1959) has suggested that "the response to GA alone by tissues with high endogenous growth rate is thus probably attributable, as is also the high endogenous growth rate itself, to the presence of residual endogenous auxin". This conception of the mechanism relating endogenous and gibberellin-induced growth is, however, questionable in view of the results of Purves and Hillman (1958) and those described in the present paper (Figure 2). When sections with low endogenous growth rates are given sufficient IAA to match the endogenous growth rates of more apical sections, the addition of GA still produces less additional growth than in the latter. Therefore, the relationship between endogenous and gibberellin-induced growth must be explained in other terms. This is not surprising, since the older, less rapidly elongating tissue is anatomically different from the younger tissue; a single factor could hardly account for the difference in growth rates.

"Auxin-Sparing" by Gibberellin

Galston (1959), Pilet (1957), and Stutz and Watanabe (1957) have shown that GA treatment results in a decreased IAA-oxidase activity in certain tissues. McCune and Galston (1959) found that the peroxidase activities of dwarf corn leaf sheaths and dwarf pea internodes were decreased by GA treatment. On the other hand, Brian and Hemming (1958) and Kato and Katsumi (1958) found no effects of GA on IAA-oxidase activities, and Hayashi

et al. (1956) reported that GA treatment caused an increased peroxidase activity in leaf sheaths of rice. Since IAA can be destroyed *in vitro* by either IAA-oxidase or peroxidase under appropriate conditions, reductions of the activities of these systems by GA have been interpreted to support the theory that gibberellin acts through an "auxin-sparing" (auxin-protecting) mechanism. According to this theory, the growth-promoting action of gibberellin is a secondary effect which results from an increased level of native auxin.

While reports of changed IAA-oxidase and peroxidase levels are interesting, they do not compel the conclusion that gibberellin acts through an auxin-protecting mechanism. It should be borne in mind that IAA may not be the major auxin in the pea plant (Good *et al.* 1956) and that IAA-oxidase is not active on other auxins such as naphthaleneacetic acid and 2,4-dichlorophenoxyacetic acid. Also, while IAA-oxidase activity appears to be correlated with certain growth phenomena (*e.g.*, Galston 1956), there is no unequivocal evidence that this enzyme system is of importance in the intact plant (Briggs *et al.* 1955, Galston and Hillman, *in press*).

Even assuming a physiological significance for IAA-oxidase activity assayed in extracts, the interpretation of the results of Stutz and Watanabe (1957) is dubious. In their experiments, IAA-oxidase determinations were made ten days after the conclusion of an extended period of GA treatment, during which time considerable growth had occurred. In such a system, changed levels of any compounds can hardly be considered primary effects of gibberellin treatment. Also, the effects reported by Galston (1959) and by Pilet (1957) are obviously quite separate. Galston has reported that the effect of GA on the plant was to raise the level of an *in vitro* inhibitor of IAA-oxidase, while Pilet's study involved the addition of GA to an *in vitro* IAA-oxidase preparation so that the effect of GA must have been a direct one upon the enzyme system itself. Since these reports of auxin-protection differ from each other, and since other investigators have failed to observe similar effects in other systems, these effects of GA on IAA-destroying systems may be secondary ones and not responsible for the growth-promoting activity of GA.

Nitsch (1957) demonstrated that treatment of *Rhus typhina* with GA resulted in an increased level of native auxin. This finding is consistent with an auxin-protecting mechanism of gibberellin action. It remains to be shown, however, that the change in auxin occurs before and contributes to the increased growth rate, rather than taking place as a result of the changed growth. Hayashi and Murakami (1958 a) observed no effect of GA on auxin levels in several species.

If gibberellin acts by virtue of an auxin-protecting mechanism, it should promote growth only in the presence of suboptimal auxin concentrations. When supraoptimal (inhibitory) levels of auxin are available, the addition

of gibberellin should result in a further inhibition of growth according to this theory. The data of Figure 3 show that this is not so, and appear to be particularly destructive to the concept of an auxin-protecting mechanism of gibberellin action. Other reports also bear on this hypothesis, since it suggests that GA should inhibit the growth of roots, promote the initiation of adventitious roots, and inhibit the growth of lateral buds, all effects characteristic of IAA. However, it has been shown that GA does not inhibit the growth of roots, even in the presence of inhibitory IAA levels (Brian *et al.* 1955); also, GA inhibits root initiation (Brian *et al.* 1955, Kato 1958) and promotes lateral bud growth (Brian *et al.* 1955, Kato 1953, 1958, Wickson and Thimann 1958).

Other Mechanisms of Auxin-Mediated Gibberellin Action

While gibberellin probably does not act through an auxin-protecting mechanism, other close relationships between the actions of gibberellin and auxin may be envisioned. For instance, gibberellin might promote growth by antagonizing an inhibitor of auxin-induced growth (Brian and Hemming 1958, Galston and Warburg 1959). Mechanisms such as this are not opposed by the data cited above. However, other data presented appear to be inconsistent with any mechanism involving auxin-mediated gibberellin action. Treatments which affect the auxin relations of pea sections show no such effects on the response of the sections to GA. For instance, Purves (1959) has shown that the addition of 2 % sucrose to the growth medium produces profound changes in the IAA dose-response curve. On the other hand, the interaction of GA with sucrose involves only a synergism (Figure 1). Other treatments which markedly alter the auxin responses of the sections are without any effect on the GA responses. Decapitation of seedlings before the harvesting of sections lowers the control growth and alters the auxin response drastically (Figure 5), and similar results are obtained when sections are treated with PCIB (Figure 4); the GA responses are not affected by these treatments. Similar results have been obtained by Applegate (1957). He treated seedlings of *Zinnia elegans* with GA and 2,3,5-triiodobenzoic acid (TIBA), which interferes with the polar transport of auxin (Niedergang-Kamien and Skoog 1956). Treatment with TIBA, while it inhibited growth (and, presumably, markedly altered the auxin relations of the internodes), did not affect the GA-induced internodal elongation. All of these data seem particularly prejudicial to the concept of an auxin-mediated mechanism of gibberellin action. They do not contradict the conclusions of Brian and Hemming (1957, 1958) and of Kuse (1958), who hold that auxin is necessary for the occurrence of a gibberellin response. They do, however, make it dif-

difficult to envision a mechanism, consistent with these findings, whereby either auxin or gibberellin could owe its action to a primary effect on the other's metabolism or mode of action.

Gibberellin-Auxin Synergisms

Gibberellin-auxin synergisms in the promotion of growth have been reported by several workers. These include interactions in the promotion of elongation, reported by Asprey *et al.* (1958), Brian and Hemming (1957, 1958), Galston and Warburg (1959), Hayashi and Murakami (1953, 1958 b), Kuse (1958), Radley (1958), Weijer (1959), and Whaley and Kephart (1957). Synergisms in other systems have been reported by Dure and Jensen (1957), Luckwill (in press), Schroeder and Spector (1957), and Wareing (1958). In the systems described by Brian and Hemming and by Kuse, gibberellin was inactive in the absence of exogenously supplied auxin. Such findings do not necessitate an auxin-mediated mechanism. An alternative explanation might simply be that the growth of these systems is limited by auxin.

Certain of the reported GA-IAA synergisms deserve further consideration. Weijer (1959) has claimed that GA and IAA are synergistic in promoting the growth of *Impatiens balsamina*, but this conclusion does not appear to be supported by his data. The further conclusion that GA acts by "neutralization of auxin inhibitors" appears to result from an overextension of a minimum of data. Hayashi and Murakami (1953, 1958 b) have reported a GA-IAA synergism in the growth of starved pea sections, but this finding could not be repeated in the present study.

Galston and Warburg (1959) have reported that GA transported through pea epicotyl segments was synergistic with IAA supplied to sections excised from the segments treated with GA. They further reported that the magnitude of the GA response was dependent upon the length of the epicotyl through which the GA was transported. Neither of these findings was confirmed in the present study. The disagreement over the reported synergism rests, however, on interpretation rather than experimentation. In computing the magnitude of the synergism, Galston and Warburg compared the combined effect of GA given *through the epicotyl* and IAA given to sections with the sum of the effects of IAA and GA given separately *to the sections*. But the proper control for the effect of gibberellin might rather be the growth induced by GA given through the epicotyl rather than by GA supplied to the sections since, in the combined treatment, the GA is supplied through the epicotyl. If this criterion is employed, no GA-IAA synergism is found in the data of Galston and Warburg.

It is probable that, while the actions of auxin and gibberellin are separate,

the two actions are mutually dependent, since the normal growth of a plant cell is the result of many processes. While it is possible that all of the changes in the cell are mediated by one "master reaction" and that this reaction is primarily controlled by one factor, such as auxin, another possibility is at least as likely. This possibility is that different changes are primarily mediated by different factors. An interdependence of the effects of these factors results from the fact that maximum growth is not attainable without the participation of all of the component cellular changes. Thus, the gibberellin-auxin interactions which have been observed are probably only secondary, and, on the evidence so far, no more intimate than the innumerable interactions which can be observed between growth substances, minerals, or other factors applied to the same organism.

Summary

Etiolated pea epicotyl section growth tests have been used to test the hypothesis that gibberellin acts through an auxin-mediated mechanism. GA promotes elongation even in the presence of inhibitory levels of IAA. Decapitation of seedlings before harvesting sections results in a decrease in control growth and markedly alters the response of sections to IAA, but it has no effect upon the GA response. Similarly, α -(*p*-chlorophenoxy)-*iso*-butyric acid reduces the control growth and alters the IAA response but does not affect the GA response. These results, along with a critical consideration of the literature, suggest that the primary actions of gibberellin and auxin are not closely connected.

Sections with a low control growth rate (5 mm. from the apex) have a lower GA response than more apical, rapidly growing sections, even when IAA is supplied to increase the "control" growth; this shows that the relationship between control growth and GA response cannot be explained in terms of auxin relations alone.

When GA and IAA are supplied simultaneously, the growth responses are partly additive; synergism is never observed, even when GA is transported to the section through an epicotyl segment. The growth of starved sections is promoted by either GA or IAA alone, and no synergism is found.

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Photoperiodic and Thermoperiodic Control of Indoleacetic Acid Oxidase in *Lupinus albus* L.

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The relation of auxins to photoperiodic induction is a well established fact. Therefore, a study of the eventual effect of photo- and thermoperiodic impulses on the activity of indoleacetic acid (IAA) oxidase seemed to be warranted. Studies concerning this problem have recently been conducted by Stutz and Watanabe (30). They came to the conclusion that the activity of IAA-oxidase in extracts from stems of *Lupinus albus* exposed to long days (16 hr illumination) is preceded by a lag phase in contrast to the immediate enzyme-activity in homogenates from plants held under non-inductive circumstances. In the present paper the results of Stutz and Watanabe (30) are partly confirmed and a number of further data, relating to the problem outlined above, will be presented.

Materials and Methods

Lupinus albus L., variety "Gyulatanyai édes" was used throughout the experiments. The photoperiodic treatment was carried out by exposing the plants to direct sunlight or to an illumination of low or high intensity from filament lamps. Details of these experiments are summarized in Table 1. It may be seen that long-day treatments consisted of continuous illumination. Thermoperiodic treatments were carried out only in the short-day variant of the second treatment by decreasing the night-temperature from 25° to 17° and 10°C respectively. Pfeffer nutrient solution was used (7, 20) and special experiments have been conducted to show that the N and P content of the nutrient solution affected IAA oxidase activity only if applied in extremely low or high concentrations (8).

Table 1. *Treatment of the experimental plants.*

No. of treatment	Source of nutrients	Treatment during the light period (8h)		Treatment during the dark period (16h)
		Light source	Light intensity (lux)	
1	Garden soil, in pots	Direct daylight	70.000	Short day: darkness Long day: 2.000 lux
2	Pfeffersolution	Filament lamp	9.000	Short day: darkness Long day: 9.000 lux
3	Pfeffersolution	Filament lamp	2.000	Short day: darkness Long day: 2.000 lux

Plants for the assays have been selected when the 2 first leaves were fully developed and the primordia of the third leaf also appeared. The fully developed leaves were then homogenized in a porcellan mortar and the homogenate pressed through a cheesecloth. This juice is called "100 % extract" in the subsequent discussions. When the effect of dilution on enzyme activity was studied the extract has been diluted with distilled water. Dialysis and the application of 2,4-dichlorophenol (DCP) was carried out as described by Stutz (29).

IAA oxidase activity was estimated by *a.* manometric procedures in a Warburg apparatus according to Stutz (29) on the basis of increase in O_2 -consumption upon addition of substrate at 30°C, and *b.* paper chromatography. In this latter case the solutions contained by the Warburg flasks were removed, boiled for 5 minutes, filtered and 25 μ l of the clear filtrate assayed by paper chromatography (paper Macherey-Nagel No. 214) after an equilibration for 14 hrs. The solvent system used was n-butanol : ammonia : water (10 : 1 : 1, 19). The chromatograms were run in the dark at room temperature (ascending technique). When the front reached a distance of 24 cm from the start line the chromatograms were dried at room temperature for 14 hrs, and evaluated under UV light or sprayed with Gordon-Weber reagent (10). As may be seen in Figure 1. the spot above IAA, its oxidation product, is strongly fluorescent and gives a feeble reaction with the Gordon-Weber reagent, in contrast to the experiments conducted with the "Omphalia enzyme" resulting in IAA oxidation products exhibiting a completely negativ reaction (28).

Only the results achieved by the Warburg technique will be included in this paper. It is stressed, however, that the paper chromatographic studies (quantitative evaluation of the spots in UV light by planimetry) always yielded analogous results.

The experiments have been repeated 5 times in 3—3 series. The results were reproducible within the limits of the experimental error referred to in the text.

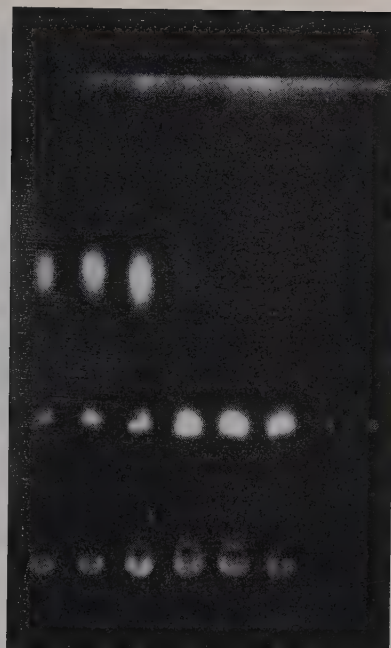
Results

As may be seen from Table 1 plants in the "first treatment" received an 8 hr illumination by direct sunlight (70.000 lux). One series of these plants was kept in darkness during night (short day) whereas the other series received a supplementary illumination of 2000 lux and was, thus, exposed

Figure 1. *Demonstration of IAA oxidase activity by paper chromatography.* Spots in the left row: IAA incubated with lupine extract for 90 min. Spots in the right row: IAA incubated with boiled lupine extract for 90 min.

From the bottom: Start (contaminations)
Rf 0.31 IAA
Rf 0.62 oxidized derivate

At the top: Front.



to continuous light-effect. The long-day plants of the "second treatment" received 9000 lux for 24 hrs, whereas the short-day plants were kept after an illumination for 8 hrs in darkness (16 hrs). The "third treatment" was the same as the second one but the plants were given in illumination of only 2000 lux.

Table 2. *Dependence of weight and length of 21 days old Lupinus albus plants on the photoperiodic treatment (illumination: 9000 lux).*

Plant organs	Dayly illumination							
	8h				24h			
	Fresh weight (g)	Dry weight (%)	Length (mm)	σ	Fresh weight (g)	Dry weight (%)	Length (mm)	σ
Root.	0.41	5.86	144.2	19.7	0.40	6.23	138.6	16.7
Hypocotyl	0.51	6.28	54.5	11.6	0.68	6.61	74.4	9.4
Cotyledons . . .	0.82	7.31	—	—	0.71	7.60	—	—
Stem.	0.11	6.25	18.8	5.9	0.23	6.09	45.3	6.4
Petiole	0.15	7.31	58.7	6.3	0.17	8.20	64.2	8.0
Leaf blade . . .	0.40	9.50	—	—	0.46	9.11	—	—
Sum	2.40	7.15	276.2	—	2.65	7.30	322.5	—

NB. 1. σ -values refer to the length.

2. The difference between the length of hypocotyl and stem is significant.



Figure 2. 4 weeks old *Lupinus albus* plants showing the effect of various photoperiodic treatments. Left: 8 hrs direct daylight, 16 hrs darkness. Right: 8 hrs direct daylight, 16 hrs supplementary illumination (2000 lux).

First treatment. The experimental plants reached the flowering stage only in case of the first treatment. Under continuous illumination flowering of the variety used started 28—30 days earlier than under short-day treatment and the appearance of flowers was preceded by the development of a lesser number of leaves (8). These experiments seemed to be necessary since *Lupinus albus* is considered by some workers as a long-day (14, 31) by others, however, as a short-day (12, 27) plant. The striking temperature dependence of the developmental processes of *Lupinus albus* was also emphasized by a number of authors (25, 32). Long-day treatment resulted in a marked increase in length as already shown by Németh (19). Four-week old individuals may be seen in Figure 2.

The auxin oxidase activity from homogenates (30 %) of leaf tissues is shown in Figure 3.

The σ -values in these and in the subsequent experiments ranged between 0.77 and 0.83. Therefore a difference of 5 % is significant.

It may be seen that no IAA-oxidase activity could be detected in extracts from plants exposed to long-day treatments. By contrast, the IAA oxidase activity was shown to be high in plants grown under short-day conditions.

Figure 3. IAA oxidase activity of short-day (1) and long-day (2) *Lupinus albus* plants. Illumination by direct sunlight. Abscisse: time of incubation in minutes. Ordinate: consumption of O_2 in $mm^3/1$ mg dry matter.

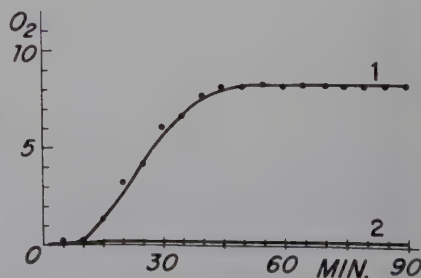
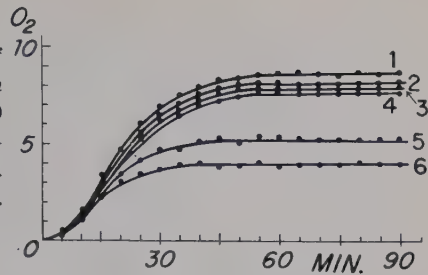


Figure 4. *Dependence of IAA oxidase activity of short-day treated lupine leaves on the concentration of the extract.* Illumination by artificial light: 9000 lux. Abscisse: time of incubation in minutes. Ordinate: consumption of O_2 in $mm^3/1$ mg dry matter. Concentration of extract: 1. 100 %, 2. 88 %, 3. 75 %, 4. 50 %, 5. 33 %, 6. 25 %.



Second treatment. The size and dry weight of short-day and long-day plants differed depending on the photoperiodic treatment. The plants kept on constant light elongated but the differences were not as striking as those obtained in the first treatment by direct sunlight. Some characteristic data of the plants are summarized in Table 2.

The IAA oxidase activity of the homogenates from leaves is indicated in Figures 4 and 5.

It may be seen that the enzyme activity of short-day treated plants is dependent, of course, on the concentration of the extracts; however, no lag-phase precedes the operation of IAA oxidase in the system. On the contrary, long-day treated plants yielded extracts exhibiting a pronounced lag-phase in IAA oxidase assays, which depended on the concentration applied. A representative example is shown in Figure 5. The results have shown always the same general trend. In some cases the lag-phase of the 100 percent extract lasted up to the end of the incubation period (180 min.). As to the nature of the lag-phase we maintain the opinion that it is due to the presence of some inhibitory substances. The opinion is supported by the observation that the length of the lag-phase could be extended by the addition of DCP to the system (Table 3).

It is interesting to note that the activity of the enzyme is not lowered, only the lag-phase is extended by the addition of DCP. In some cases the lag-

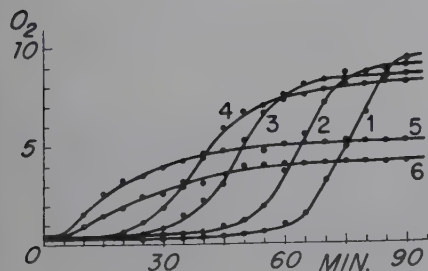


Figure 5. *Dependence of IAA oxidase activity of long-day treated lupine leaves on the concentration of the extract.* Illumination by artificial light, 9000 lux. Abscisse: time of incubation in minutes. Ordinate: consumption of O_2 in $mm^3/1$ mg dry matter. Concentration of extract: 1. 100 %, 2. 88 %, 3. 75 %, 4. 50 %, 5. 33 %, 6. 25 %.

Table 3. *Dependence of the lag-phase of IAA oxidase upon the daily illumination, the concentration of the extract and the presence of DCP. Concentration of DCP: 0.2 μ M/ml in the reaction mixture. Length of the lag-phase in minutes.*

Extract %	Daily illumination			
	8h		24h	
	IAA+H ₂ O	IAA+DCP	IAA+H ₂ O	IAA+DCP
100	13	15	65	144
88	12	14	57	142
75	12	14	40	120
50	11	15	28	65
33	12	12	13	25
25	12	12	12	18

phase might be "indefinitely long", leading to qualitative differences between short-day and long day treated plants. This seems to be the case with plants that received direct daylight.

It should be born in mind that DCP generally stimulates the IAA oxidase activity. Experiments on the effect of dialysis on enzyme activity have really shown that the IAA oxidase activity lost upon dialysis can be restored by the addition of DCP. The inhibitory effect of DCP on crude systems is not surprising. As reported by Reinert *et al.* (26) the activity of IAA oxidase can be stimulated or inhibited by the same compound depending on the experimental conditions.

The results of our experiments on thermoperiodism are summarized in Table 4.

It may be seen that if the temperature during illumination and darkening is kept on the same value a lag-phase appears in the extracts of short day plants as well. This lag-phase is, however, always shorter than that exhibited by extracts from long-day treated plants. Probably the lower night temperature do not favour the synthesis of the inhibitor.

Third treatment (see Table 1). The size of plants was independent from the photoperiodic treatment. IAA oxidase activity was experienced in both short-day and long-day treated plants without the appearance of a lag phase. The enzyme activity was practically the same as in plants of the 1st and 2nd treatments.

On the basis of all that has been said above we came to the conclusion that the photo- and thermoperiodic stimuli affect the operation of IAA oxidase probably via the control of an inhibitor. High light intensity and high night temperature favour the synthesis of inhibitor.

Table 4. *Effect of night temperature on the lag-phase of IAA oxidase.*

Duration of illumination in hrs.	Day temp. °C	Night temp. °C	Duration of the lag period of a 100 % extract in minutes
24	25	25	60—∞
8	25	25	0—75
8	25	17	0
8	25	10	0

Discussion

Much attention has been paid recently to the relation of auxin metabolism to flowering. It has been demonstrated that the auxin level is decreased under the effect of short days, whereas long days favour an increase in endogenous auxin concentration (1, 3, 15, 17). Liverman and Lang (18) succeeded in inducing flowering in long-day plants kept under short-day conditions by auxin treatment. The present study revealed that the activity of auxin oxidase is inhibited in long-day treated plants leading probably to an elevated auxin level. This conclusion is justified by the experiments of Pilet indicating a direct relationship between auxin content and IAA oxidase activity in plant tissues (21). Furthermore Galston and Dalberg (5) pointed out that no auxin oxidase can be detected in rapidly growing plant tissues. The results reported in this paper are in line with the literary data mentioned above as an inhibition of IAA oxidase activity could be demonstrated in rapidly growing long-day treated plants. It is very likely that the inhibition of enzyme activity is due to the presence of an inhibitor which might be regarded as a "third factor" playing a role, in addition to auxin and gibberellins, in the developmental processes of higher plants. This idea is supported by the observation that flowering of long-day plants could be induced under short-day conditions if the dark period, at a suitable moment, was interrupted by illumination with red light (17). Hillman and Galston (13) revealed that the IAA oxidase activity is inhibited by red light and the inhibition can be abolished by illumination with infrared. The red-infrared interaction on the activity of IAA oxidase is, therefore, the same as the effect of red and infrared light on plant development. The relation of light to auxin oxidase is also indicated by further experiments (4).

It seems likely that the inhibitor, the existence of which is suggested by the experiments reported in this paper, is identical with the so called "third factor" which would play, according to the present day theories, an important role in the auxin-gibberellin interactions. The gibberellins exert an inhibitory effect *in vivo* on auxin oxidase activity (22, 24, 30). *In vitro*, however, they do not affect the IAA oxidase (11). Evidently the inhibition takes place via

a third substance as suggested by Brian and Hemming (2) and later by Galston and Warburg (6). According to the latter workers the "auxin sparing" effect of gibberellins is probably mediated by an auxin oxidase inhibitor (6). Our experiments suggest that such an "auxin sparing" inhibitor is formed under long-day conditions. As to the chemical nature of the inhibitor only suggestions can be made for the present. According to all probability a phenolic compound is involved. The same opinion is also maintained by Gortner and Kent (11) and Pilet and Galston (23). Coumarin derivatives are known to act on auxin oxidase (11) and the stimulation of flowering achieved by Leopold and Thimann (16) by the application of coumarin may possibly be interpreted as being due to a primary effect on auxin oxidase.

Summary

1. The activity of auxin oxidase is influenced by the photoperiodic conditions. The effect is probably based on the synthesis of an enzyme inhibitor in long-day treated plants.

2. Intense illumination favours the development of the hypothetic inhibitor as can be judged from the length of the lag-phase of IAA oxidase activity. When, however, the illumination is as low as 2000 lux, no lag phase is to be observed.

3. When the temperature during the dark period is lowered the lag phase is shorter, apparently because of a lower level of inhibitor synthesized.

4. As discussed in detail, the data presented support the hypothesis that the inhibitor observed might be identical with the hypothetic "third factor" the existence of which has been postulated on the basis of studies on auxin-gibberellin interactions.

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Effect of Osmotic Concentration on Auxin-action and on Irreversible and Reversible Expansion of the *Avena* Coleoptile

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In 1931 Heyn suggested that auxin induces growth in *Avena* coleoptiles by causing an increased plasticity of their cell walls and presented data to support this suggestion. In subsequent years this theory became discredited and attention was diverted to other possible mechanisms. Recently new evidence has been brought forward which indicates that auxin does cause an increase in cell wall plasticity in *Avena* coleoptiles (Kobayashi *et al.* 1956, Tagawa and Bonner 1957, Cleland 1958) although the physical nature of the plastic extension appears to be different from that envisaged by Heyn. Evidence which apparently contradicts this mechanism has been presented by Pohl, however. He has shown (1957) that if the growth rate of auxin-treated sections is reduced to that of the non-auxin-treated controls, both sets of sections will attain the same final length when they are placed in an isotonic solution. If auxin causes a permanent loosening of the cell wall, the auxin-treated sections should have the greater length when both sets of sections are in a solution of the same osmotic concentration. The possibility exists, however, that the cell wall loosening is not a permanent process but is dependent upon the turgor pressure of the tissue and only manifests itself in hypotonic solutions. To test this hypothesis, the effect of the osmotic value of the solution (O_{Pe}) upon the process of auxin-induced irreversible expansion of *Avena* coleoptiles has been determined.

Although some determinations of the relationship between O_{Pe} and total expansion have been carried out by Thimann and Schneider (1938), Ketellap-

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per (1953), Bennet-Clark (1956), and Pohl (1953), only Ordin *et al.* (1956) have examined the relation between OPe and irreversible expansion (IE). They find a non-linear relationship between OPe and IE.

There is no method for determining irreversible expansion which is free of all serious limitations. The desirability of having the driving force of expansion be the physiological process of osmotic water uptake eliminates determinations by means of external weights and mechanical bending. The most satisfactory technique is a modification of that devised by Ursprung and Blum (1924) where the length of the tissue after plasmolysis is compared before and after a period of expansion.

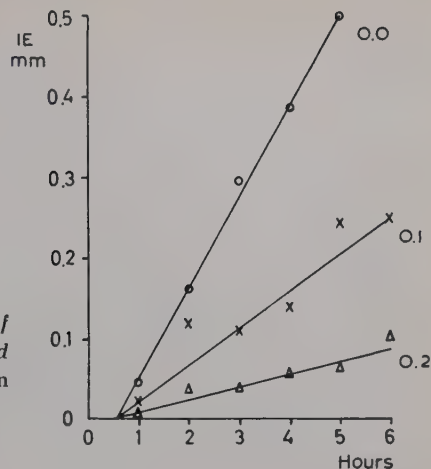
Reversible expansion (ES) can also be determined at the same time. The opportunity has been taken to determine the relation between OPe and ES, which has apparently never been determined before for *Avena* coleoptiles. This relation has been checked by employing an independent technique for determining tissue elasticity, developed by Virgin (1955). This involved measurement of the tissue tensility, which can be related to elastic stretching, by determination of the resonance frequency of the tissue when under the influence of an oscillating electrical current.

To avoid confusion, the following terms used in this paper are defined. Irreversible expansion (IE) is the increase in the plasmolyzed length of the tissue. Plastic stretching (PS) is irreversible expansion of wall already present. PS is the same as, and equal to IE only if no wall synthesis occurs. In the presence of wall synthesis PS constitutes some part of IE. Plasticity is the amount of plastic stretching which can occur in one unit length of tissue during one unit of time under the influence of one unit of turgor pressure. It should be noted that this concept of plasticity is based on the biological process of wall deformation and not on the strict physical concept of plasticity which involves "overstretching". An increase in plasticity will lead to plastic stretching, but an increase in PS can occur without an increase in plasticity. Elastic stretching (ES) is the reversible expansion of the tissue. Elasticity is the amount of elastic stretching which occurs when one unit of tissue is stretched by one unit of turgor pressure.

Materials and Methods

Oat seeds were germinated in vermiculite, moistened with distilled water. The plants were grown in a cabinet in which the temperature was kept at $25 \pm 1^\circ\text{C}$. and the humidity at $90 \pm 3\%$. After 72 hours in the dark, the plants were illuminated with a weak red light to reduce mesocotyl growth. Coleoptiles of 2.5 to 3.0 cm. in length were then selected (ca. 96 hours) and after the apical 3 mm. had been discarded, the next 5 mm. of coleoptile was removed as one section. This operation, and all subsequent operations were carried out under a weak green light. The sections were randomized by placing in distilled water until all were cut. They were then divided into lots of twenty sections. One lot was plasmolyzed by treatment in

Figure 1. Time curves for irreversible expansion of *Avena coleoptile* sections in solutions of 0, 0.1, and 0.2 *M* mannitol. All solutions are 0.0025 *M* in K-maleate (pH 4.8) and contain 5 ppm. IAA.



1 *M* mannitol for ninety minutes and their length was then determined with a microscope fitted with an eyepiece micrometer. This gave the initial plasmolyzed length (PLi). The remaining lots of sections were placed in the various experimental solutions which were 0.0025 *M* K-maleate solutions (pH 4.8) containing sufficient mannitol to produce the desired OPe and with or without IAA, 5 ppm. At the end of the expansion period, which varied from 4 to 10 hours depending upon the experiment, the length of the sections was determined (Lf). They were then plasmolyzed by treatment in 1 *M* mannitol for 90 minutes and their length again measured (PLf). The irreversible and reversible expansion were then determined by the following relations:

$$IE = PLf - PLi \text{ and } ES = Lf - PLf$$

Values of IE and ES were then plotted against OPe. The resulting curves show the manner in which IE and ES are influenced by the osmotic value of the solution.

The values of IE shown in Figures 2—8 are the amounts of irreversible expansion which occurred during the experimental period of a certain duration. IE increases linearly with time at all OPe values (Figure 1). The values of IE can be transformed into rates of irreversible expansion and the IE-OPe curves are also "rate of IE"-OPe curves. Thus it makes no difference what length of expansion period is used (up to 16 hours at least), the resulting curve will have the same shape.

The measurement of tissue elasticity by means of resonance frequencies has made use of a technique which is a slight modification of that developed by Virgin (1955). *Avena* seedlings were grown in the manner described above. Coleoptiles of 2.75—3.25 cm. length were selected, the central leaf removed, and the apical 5 mm. excised. The sections were then incubated for 6 hours in 0.0025 *M* K-maleate solutions (pH 4.8) containing sufficient mannitol to produce the desired OPe and without auxin. At the end of this period, the sections were trimmed to 15 mm. length by removing the excess basal part, placed in a vertical position by insertion of a 3 mm. stainless steel pin into the basal end of the empty leaf chamber, and the frequency necessary to produce maximum vibration was determined as described by Virgin. The resonance frequency had to be determined with the coleoptile in air because of

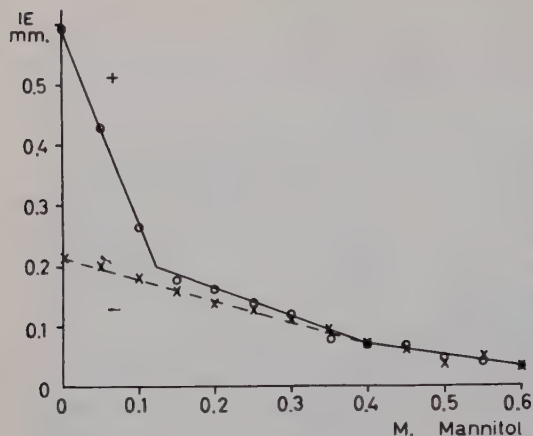


Figure 2. The effect of the osmotic concentration of the solution on the irreversible expansion of *Avena coleoptile* sections as influenced by auxin. Sections allowed to expand for 4 hours in buffered mannitol solutions of varying concentration, with (—O—) and without (—X—) IAA, 5 ppm.

the poor resolution of the oscillation when in solution. During the short time (ca. one minute) between removal of the section from the solution and determination of the frequency, dehydration caused less than a 5 % change in the resonance frequency. All points in Figure 14 are the average of at least 15 separate determinations. The square of the resonance frequency (v^2) is then plotted as a function of OPe.

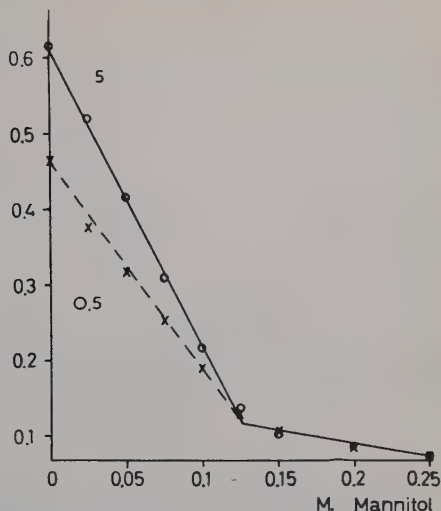
Results

In the absence of auxin, the irreversible expansion of *Avena coleoptiles* is proportional to the "water concentration" of the external solution over the whole range of OPe values between that of pure water and that causing incipient plasmolysis (ca. 0.4 *M* mannitol). The points fall in a single straight line of low slope; in the absence of auxin the tissue possesses a low potential for irreversible expansion (Figure 2).

Auxin has no appreciable effect upon the rate of irreversible expansion in the region of OPe values between 0.4 and ca. 0.15 *M* mannitol. When OPe is decreased below some critical value the expansibility of the tissue suddenly becomes markedly increased by auxin; the points in this region of low OPe fall along a straight line of greater slope. The slope of this line depends upon the concentration of auxin (Figure 3). The exact position of the break or critical point between the two OPe regions varies slightly between experiments but is always in the region of 0.1 to 0.15 *M* mannitol. The break in the curve is sharp, as can be seen from Figure 4. The position of the critical point does not vary with a change in auxin concentration (Figure 3).

The procedure employed in the previous experiments only allows one to determine IE. This may be equivalent to plastic expansion (PS), but only if there is no new wall synthesis. Thus any conclusions about the manner in

Figure 3. *Effect of varying auxin concentration on the slope of the IE-O_{Pe} curve. Sections allowed to expand for 6 hours in buffered mannitol solutions of varying concentration, and with the addition of 5 (—○—) or 0.5 (—×—) ppm. IAA.*



which IE is influenced by O_{Pe} may not hold for PS. However, PS can be determined by modifying the procedure in the following manner. Sections were incubated in an isotonic mannitol solution (0.25 M) for one hour. To some sections auxin was added at the start, to others after thirty minutes and to others not at all. All three groups were then allowed to expand for 3 hours in auxin-free solutions of varying O_{Pe} and with metabolism blocked either by N₂ or by low temperature (4°C.). When PS-O_{Pe} curves were constructed, a single straight line of low slope was obtained for the non-auxin-treated controls while the auxin-treated sections showed a two-part curve

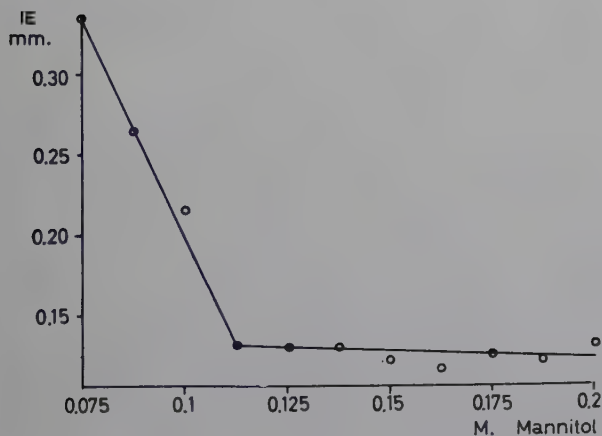


Figure 4. *Demonstration of the sharpness of the break in the IE-O_{Pe} curve. Sections allowed to expand for 6 hours in solutions of varying O_{Pe} and containing 5 ppm. IAA.*

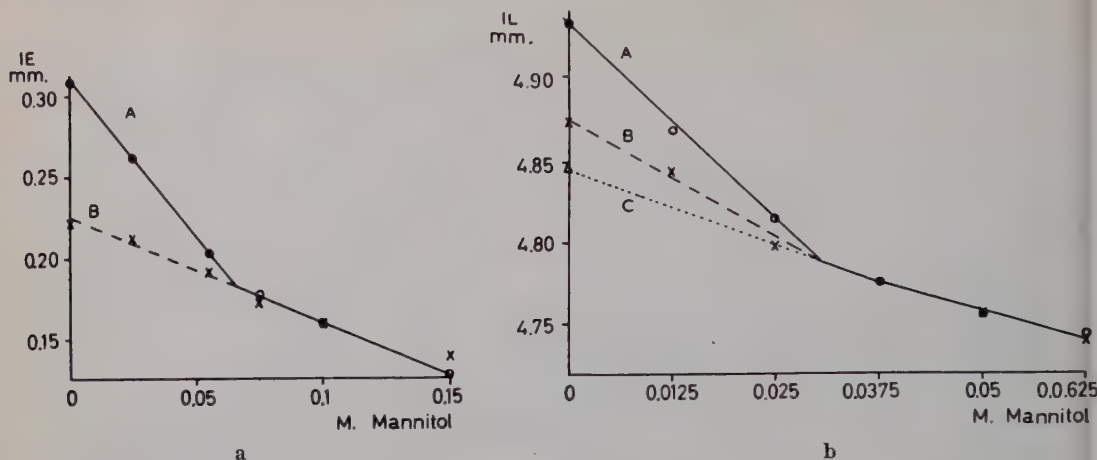


Figure 5. The effect of varying lengths of incubation with auxin on the PS-OPe curve. Sections pretreated in 0.25 M mannitol for one hour, then allowed to expand in solutions of varying OPe, containing N_2 to prevent any further auxin-action or aerobic metabolism.

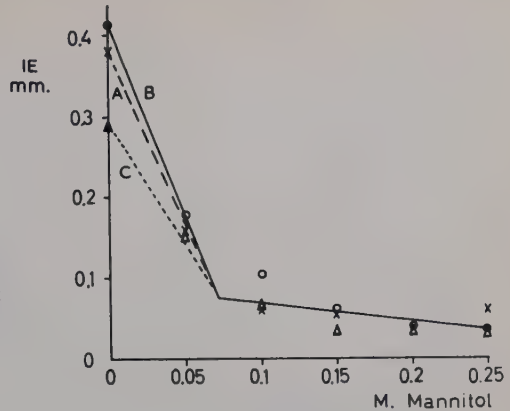
- a) Curve A, 5 ppm. IAA during hr. of pretreatment. Curve B, no auxin during pretreatment. Expansion 150 minutes.
- b) Curve A, 5 ppm. IAA during whole hour of pretreatment. Curve B, 5 ppm. IAA during last half hour of pretreatment. Curve C, no auxin during pretreatment. Expansion 120 minutes.

(Figure 5 a). The slope of the line in the region of low OPe depends upon the length of duration of auxin treatment; the position of the critical point is not affected.

These results, identical with those obtained for IE, indicate that IE and PS are influenced by auxin in the same manner and that for the purposes of this study, IE may be equated with PS. It appears that auxin can cause a loosening of the cell walls of *Avena* coleoptiles only when the turgor pressure (TP) of some group of cells exceeds some critical value.

Auxin does not cause a permanent change in the properties of the cell wall. For when the OPe is increased to a value above the critical value, the tissue acts as if it had never been exposed to auxin. This has been shown in the following manner. Sections were incubated for three hours in solutions of varying OPe (Figure 6, Curve A). Then the sections which had been expanding in pure water and whose walls were thus in a loosened state were placed in solutions of varying OPe and allowed to expand for a further three hours. The amount of irreversible expansion during this three hours was then determined and a IE-OPe curve was constructed (Figure 6, Curve B). The curve shows a characteristic critical point. When the sections originally incubated at a high OPe (0.2 M mannitol) were similarly treated, the resulting

Figure 6. Expansion of sections during incubation of 3 hr. in solutions of varying OPe and 5 ppm. IAA after varying pretreatment. Curve A ($- \times -$), no pretreatment; curve B ($- \circ -$), pretreatment for 3 hrs. in solution containing no mannitol but with 5 ppm. IAA; curve C ($- \triangle -$), pretreatment for 3 hrs. in 0.2 M mannitol solution containing 5 ppm. IAA.



IE-OPe curve was found to be almost identical in shape but of lower slope in the region of low OPe values (Curve C). The ability of a tissue to respond markedly to auxin does not depend upon whether the tissue has been loosened at some stage in its history; the osmotic value of the solution in which it finds itself determines whether the tissue is at that moment loosened or not. The amount of response of an auxin-treated tissue at any OPe will, however, depend upon the previous history of the tissue.

The position of the critical point bears no apparent relation to the state of water saturation which prevails in the tissue prior to its treatment in the experimental solutions. The water saturation of *Avena* coleoptile tissues immediately after cutting has been determined by Ordin *et al.* (1956) and Pohl (1957) as being equivalent to mannitol concentrations of 0.2 and 0.32 M, respectively. In the present studies values between 0.20 and 0.24 M mannitol have been obtained. The critical point, however, always occurs in the region of 0.1 to 0.15 M mannitol. Furthermore, it can be seen from the previous experiment that both completely water-saturated and water-starved tissues possess the same critical point when placed in the same range of osmotic solutions. The previous history of a tissue affects the quantitative but not the qualitative response of a tissue to OPe and auxin.

It has been seen that the slope of the IE-OPe curve in the region of low OPe depends upon both the concentration of auxin and the duration of auxin treatment. It is also influenced by the presence of calcium ions. This is not surprising since it has been shown that calcium and auxin-action are closely related (Burstrom 1952, Ordin *et al.* 1956, Bennet-Clark 1956, Cooil and Bonner 1957). The addition of 0.003 M CaCl_2 to the various solutions produced no effect when OPe was high but caused a decreased slope of the IE-OPe

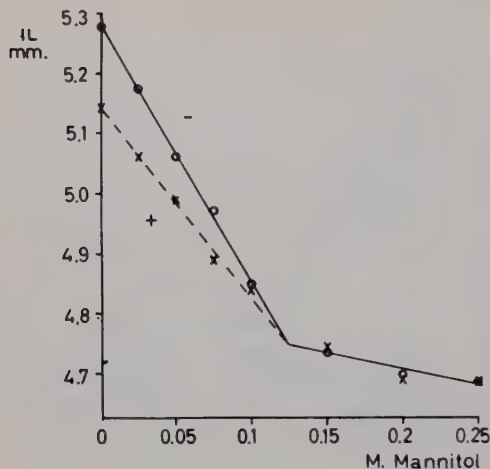


Figure 7. The effect of calcium ions on the IE-Ope curve. Sections allowed to expand for 6 hrs. in solutions of varying OPe containing 5 ppm. IAA and with (—x—) or without (—o—) 0.003 M CaCl_2 .

curve in the region of low OPe. The position of the critical point is unchanged (Figure 7).

The position of the critical point can be changed if some absorbable solute is added to the medium. It has been shown by Ordin *et al.* (1956) that addition of sucrose to the medium will cause an increase in the osmotic concentration of the cells, presumably through uptake of hexoses (Burström 1958). The addition of 1 % sucrose to the solutions has little effect upon the slope of the curves but causes a displacement of the critical point to a higher mannitol concentration than would be expected if sucrose was only acting as an osmotic agent in the external solution (Figure 8).

The curve obtained when ES is plotted as a function of OPe is found to possess two distinct regions, both in the presence and absence of auxin

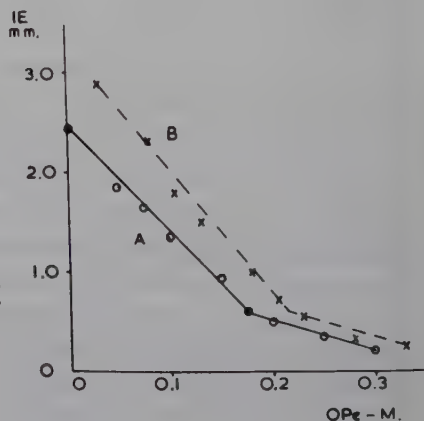


Figure 8. The effect of sucrose on the IE-Ope curve. Sections incubated $10\frac{1}{2}$ hours in solutions of varying OPe containing 5 ppm. IAA and with (B) or without (A) 1 % sucrose.

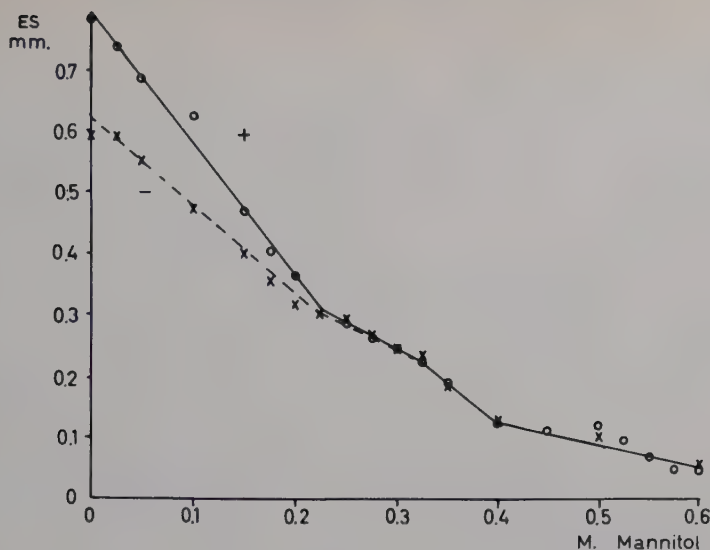


Figure 9. Relation between elastic stretching of *Avena coleoptile* sections and OPe. ES measured after expansion of 4 hrs., with (O-O) or without (X-X) IAA, 5 ppm.

(Figure 9). With concentrations of mannitol between that causing incipient plasmolysis (0.4 M) and about 0.15 M, elasticity is low. Auxin has no effect on elasticity in this region. When the mannitol concentration is decreased below some critical value, elasticity suddenly increases and becomes independent of OPe. In this region elasticity is increased by auxin. The amount of increase depends upon the concentration of auxin (Figure 10).

If total expansion is plotted as a function of OPe, the resulting curve shows two distinct break-points in some experiments (Figure 11). On separation of

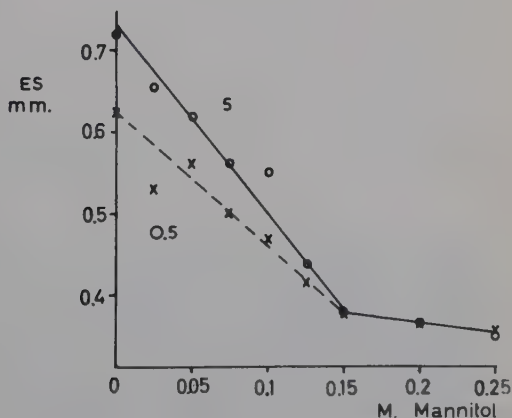


Figure 10. Influence of auxin concentration on the slope of the ES-OPe curve. ES measured after expansion of 5 hours. Auxin concentrations 5 and 0.5 ppm. (IAA).

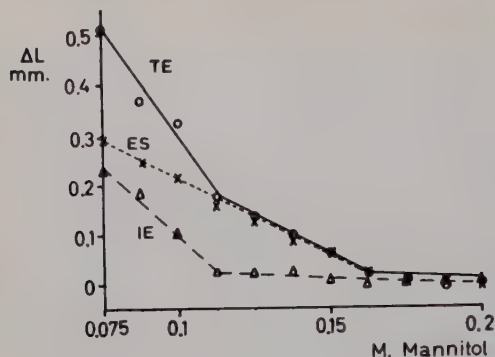


Figure 11. A demonstration of the difference in the critical points for IE and ES. Total expansion, IE and ES determined after sections had expanded for 6 hours in buffered mannitol solutions of varying OPe which contained 5 ppm. IAA. Values of TE, IE, and ES at 0.2 M mannitol taken as zero values.

one of these total expansion-OPe curves into IE- and ES-OPe curves, it is found that the critical points for IE and ES often occur at different values of OPe. If the two points do not coincide, the critical point for ES always occurs at a higher OPe.

When time curves for IE are determined, it is found that IE always increases linearly with time. This is not true for ES. When the sections are placed in the experimental solutions, there is a rapid equilibration which is finished within 30–60 minutes. Thereafter ES may change, but the rate of change will be low and will depend upon the rate of change of IE. This slow change is apparently governed by two different processes.

When plastic expansion is prevented or, at least, kept to a minimum, there appears to be a conversion of ES into IE. This is shown in Figure 12. A rapid increase in IE will be accompanied by a proportional increase in ES. The proportionality constant is determined by the concentration of auxin

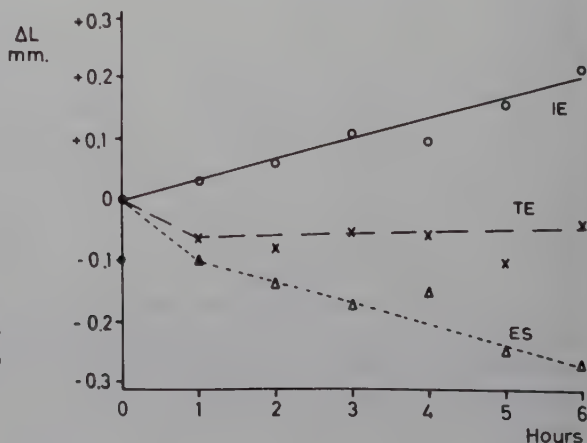
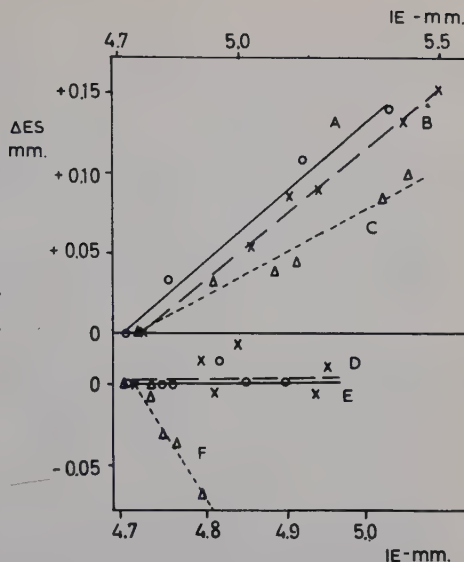


Figure 12. Conversion of ES into IE. Sections incubated in 0.2 M mannitol solution which contained 5 ppm. IAA.

Figure 13. The relation between the increases in IE and ES as determined from time curves.

- A. 5 ppm. IAA, no mannitol (—○—).
 B. 5 ppm. IAA, 0.04 M mannitol (—×—).
 C. 0.05 ppm. IAA, no mannitol (---).
 D. no auxin or mannitol (—×—).
 E. 5 ppm. IAA, 0.1 M mannitol (—○—).
 F. 5 ppm. IAA, 0.2 M mannitol (---).



rather than the rapidity of irreversible expansion (Figure 13). It is possible to obtain an OPe value in which ES remains constant after the initial equilibration. It would appear that the two processes must cancel each other in this case.

Advantage has been taken of the resonance frequency method for measuring tissue elasticity in order to confirm the relation between ES and OPe, determined by the plasmolysis method. It has been shown by Falk *et al.* (1958) that the square of the resonance frequency (v^2) is proportional to the Young's modulus of a tissue. Since Young's modulus is inversely proportional to tissue elasticity, an increase in elasticity will cause a decrease in Young's modulus. If the previous determinations of ES by plasmolysis are correct, the following curve might be expected when v^2 is plotted against OPe. In the region of high OPe, the curve should be a straight line of large slope. When OPe becomes less than the critical value, the curve should change to another straight line of lower slope. When the experiment is performed, such results are obtained (Figure 14). Both methods give identical positions for the critical point.

Bending experiments have indicated that auxin has no effect on elasticity in the absence of normal osmotic expansion. This has been confirmed in the following manner. Sections were incubated for one hour in a 0.25 M mannitol solution with or without 5 ppm. IAA. It has been shown that the auxin-treated sections then show greater plasticity when measured by bending (Cleland 1958) or expansion (Cleland and Bonner 1956). Both sets of sections

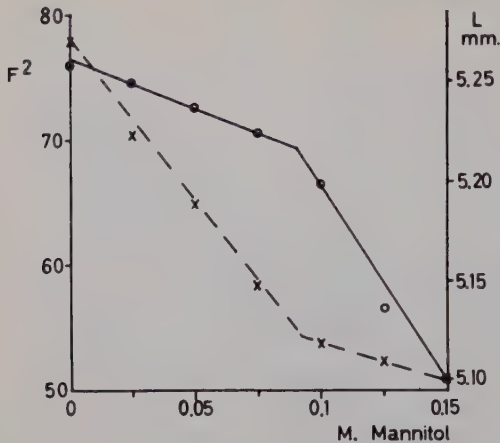


Figure 14. A comparison of the curves for elastic stretching, determined by plasmolysis (curve A) and tissue rigidity, determined by frequency of oscillation (curve B). Sections allowed to expand for 4 hours in solutions of varying OPe. ES and v^2 then determined as detailed in text.

gave the same resonance frequency, however. This indicates that auxin has no effect on elasticity in the absence of expansion, a result which is in agreement with the results of earlier experiments (Heyn 1931, Söding 1934, Cleland 1958).

Discussion

The effect of auxin on irreversible expansion depends upon the osmotic value of the solution. In solutions of high OPe the tissues possess a low potential for irreversible expansion and auxin has little or no effect upon this potential (under the experimental conditions employed). Non-auxin-treated tissues possess the same low potential even in regions of low OPe; the IE-OPe curve is a single straight line of low slope. With auxin-treated tissues, however, if OPe is below some critical value the tissues have a much greater potential; auxin induces expansion only in solutions of low OPe.

The effect of OPe on expansion has been examined before. Only Ordin *et al.* (1956) have separated IE from total expansion in coleoptiles. They obtained results with *Avena* coleoptiles which are very similar to the present ones but drew a smooth curve through the points when two straight lines would be equally justified if one point is discarded as being in error. There can be no doubt that a sharp break point between the regions of high and low OPe does occur as it has been repeatedly obtained in the present investigation. Bennet-Clark (1956) has presented time-growth curves for *Avena* coleoptile sections at various OPe concentrations. Growth-OPe curves constructed from this data are identical with those presented in this paper. Thimann and Schneider (1938) have measured the effect of OPe on extension of *Avena*

coleoptile sections incubated for 36 hours in the presence of auxin and sucrose. They found a break in the curve at about 0.4 *M* mannitol, the highest OPe which they used, and ascribe it to incipient plasmolysis. As it has been shown that sucrose will shift the critical point in the direction of a higher OPe value (through uptake of hexoses), it may be that this break-point is not due to incipient plasmolysis but is a true critical point. Ketellapper (1953) and Pohl (1953), using *Avena* coleoptiles, and Hasman (1943), employing potato tuber discs, have presented data which are consistent with the present results although in no case was there sufficient data to allow definite growth-OPe curves to be constructed.

There can be little doubt that auxin can cause an increase in the plasticity of *Avena* coleoptile cell walls. This was originally shown by Heyn (1931) and has been confirmed in recent years by Kobayashi *et al.* (1956), Tagawa and Bonner (1957), Cleland (1958) and Adamson and Adamson (1959). In most of the present experiments IE rather than PS was determined. In some experiments, however, the procedure was modified so that any expansion must be a plastic expansion. It has been shown that PS is influenced by OPe in the same manner as is IE; the effect of auxin on IE is principally due to an effect on PS.

Auxin acts upon *Avena* coleoptile tissues only when OPe is less than some critical value. The change in auxin sensitivity must actually be in response to a certain critical value of turgor pressure rather than OPe. Thus it would be desirable to convert values of OPe into TP values.

TP can be computed from the change in cell volume if the cell comes to equilibrium and if there is no osmoregulation. However, the sections do not come to equilibrium during the expansion but continue to expand at a constant rate up to at least 16 hours. Although the data are not conclusive, it appears from the work of Ordin *et al.* (1956) that osmoregulation does occur in the *Avena* coleoptile under the conditions used in the present experiments. It would be incorrect to attempt to compute TP values without more data. An indication of the relation between TP and OPe can be obtained from the resonance frequency experiments. It has been shown theoretically by Nilsson *et al.* (1958) and experimentally for potato parenchyma by Falk *et al.* (1958) that when v^2 is plotted against TP, a straight line is obtained. In the present studies, v^2 *vs.* OPe gives a straight line both above and below the critical value of OPe. Since v^2 *vs.* TP should be a straight line, TP must be directly proportional to OPe.

TP values cannot be directly computed from the present data. However, TP will be inversely related to OPe, probably linearly. Any decrease in OPe will be matched by an increase in TP.

The present results, then, would indicate that auxin only affects the plastic extension of *Avena* coleoptile tissues when the turgor pressure of some group of cells exceeds some critical value. The finding of Pohl that auxin does

not affect the wall pressure of *Avena* coleoptile sections while in an isotonic mannitol solution would be expected.

The elasticity of *Avena* coleoptile tissues has been found to depend upon the osmotic value of the solution. Under conditions of high OPe, elasticity is low. When OPe becomes less than some critical value, elasticity suddenly increases. Auxin does not affect elasticity in the region of high OPe, but in the region of low OPe elasticity is increased by auxin.

ES-OPe curves have apparently never been determined before for *Avena* coleoptiles. Such curves have been constructed by Frey-Wyssling (1950) and Burström (1953) for wheat root epidermal cells. These do not show a critical point. This phenomenon may only occur in the *Avena* coleoptile.

In what manner are plastic and elastic expansion interrelated? It was originally proposed that plastic expansion only occurred when the cells were stretched beyond some elastic limit (Heyn 1931). This concept of "overstretching" has been severely criticised by Burström (1942) and Söding (1952). Evidence that such a process does not lead to PS in *Avena* coleoptiles is given by the fact that PS occurs at all OPe values below incipient plasmolysis.

Growth might occur through a conversion of ES into IE. (Burström (1957) has suggested that inhibition of wheat root growth by auxin is due to a conversion of IE into ES.) Auxin-induced growth of *Avena* coleoptiles does not involve such a conversion. But evidence has been presented that an auxin-insensitive conversion of ES into IE does occur. It may be that this process is analogous to the "stiffening" reaction reported by Heyn (1931), Cleland and Bonner (1956) and Adamson and Adamson (1957). In none of these cases would the experimental procedure permit a determination of whether the "stiffening" consisted of a conversion of ES into IE.

In the presence of rapid, auxin-induced irreversible expansion, ES increases. This is probably due to an increase in elasticity although an increase in the osmotic pressure of the cell cannot be ruled out. The amount of increase is proportional to the amount of IE but the proportionality constant depends upon the auxin concentration.

These results all suggest a close correlation between ES and IE. However, the fact that the critical point in the ES-OPe and IE-OPe curves occur at different OPe values suggests that tissue elasticity and plasticity are governed by different parts of the cell wall or by different cell layers.

The interrelation between ES and IE makes it difficult to decide whether auxin directly influences ES, IE, or both. It has been shown that auxin has no effect on the elasticity of tissues incubated in a hypertonic mannitol solution, even though auxin causes an increase in the potential for irreversible expansion (Cleland 1958). The evidence favors the view that auxin causes

an increased plasticity of *Avena* coleoptile tissues and that as the plasticity is converted into irreversible expansion, elasticity also increases.

What is the significance of the critical point, where the response of the tissue to auxin and to OPe changes? There are two possibilities. This point could be a reflection of a change in the tensions which must occur in the tissue between the epidermal and parenchyma cells. At the present time, it is not known what role these tensions play in the process of expansion. Alternatively, this point may not be a reflection of changes between cells but of changes which occur within one group of cells whose properties control the process of elongation. If this latter situation prevails, the following hypothesis might give an explanation of the present results.

Avena coleoptile cell walls have a potential plasticity which is as great or greater than that observed under conditions of maximum auxin-induced expansion. Walls rarely attain this plasticity because they are subject to stiffening by two sets of bonds. First there is a set of bonds which are too strong to be ruptured by turgor pressure. Non-auxin-treated walls have large numbers of these bonds and thus a low plasticity. These bonds are broken by auxin, leaving a second weaker set of bonds which keep the wall stiffened. However, whenever TP exceeds some critical value, the bonds are ruptured and the wall is plastic. If TP decreases below the critical value, the weak bonds immediately reform and the wall is again stiffened.

The wall may be thought of as consisting of a large number of discrete areas each of which contains one of the strong bonds and one or more of the weaker bonds. These units must be uniformly distributed over the wall since it has been shown that expansion is uniform over the whole cell length both with epidermal cells (Castle 1955) and parenchyma cells (Wardrop 1955, 1956). Each unit is either in a plastic (both bonds broken) or a stiffened state (at least one bond intact). Wall plasticity is the sum of the number of such sites in a plastic condition. Changes in concentration or duration of auxin will cause changes in the number of sites affected and thus the plasticity without affecting the force necessary to break the weak bonds (critical point).

Elasticity would be governed by only one set of bonds. These are weak and are broken if TP exceeds a certain limit. Auxin has no direct effect on wall elasticity. However, when the wall becomes thinned out during the course of auxin-induced plastic expansion, elasticity will increase. The amount of this increase will be proportional to the amount of wall which is distended. Since the amount of wall distended is governed by the concentration and duration of auxin, it is to be expected that the increase in elasticity will also be dependent upon these factors.

These results present one final problem. The OPe value at the critical

point is not identical with that of the isotonic point of the tissues at the time of cutting. The isotonic point always occurs at a higher OPe value. This suggests that the plant normally grows under such a state of water deficiency that the turgor pressure is always subcritical and their expansion should be IAA-insensitive. Can this mean that the expansion of *Avena* coleoptiles, grown under normal conditions, is not limited by auxin?

Summary

1. Auxin-induced cell wall loosening is dependent upon the osmotic concentration of the external solution. The tissues are auxin-insensitive whenever OPe is greater than some critical value. If OPe is lowered below the critical value, auxin-induced plastic stretching occurs. The tissue again becomes auxin-insensitive if OPe exceeds the critical value. The critical value (0.1—0.15 *M* mannitol) bears no apparent relation to the isotonic value, and its position is unaffected by the auxin concentration. The rate of plastic stretching in the region of low OPe is proportional to OPe. The proportionality constant is dependent upon the auxin concentration.
2. The elastic stretching-OPe curve shows a critical point both in the presence and absence of auxin. Under conditions of high OPe, elasticity is low and auxin-insensitive, while with low OPe, elasticity is higher and is auxin-sensitive.
3. A rapid increase in irreversible elongation causes a concomitant increase in ES. The magnitude of the increase in ES is dependent upon the concentration of auxin rather than the rapidity of IE.
4. When IE is limited by a high OPe, there is an auxin-insensitive conversion of ES into IE.
5. Auxin causes a reduction in wall pressure in *Avena* coleoptiles only when turgor pressure exceeds some critical value.

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Nitrate Fertilization by Means of Ion Exchange

By

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The many studies made over the years concerning the effect of salt concentration on the growth of plants are conspicuous by the fact that in many cases the results are equivocal and often contradictory, indicating that the problem as such is of an extremely complex nature. However, in his review of research work on utilization of saline water Grillot (1954) was able to draw the following general conclusions on the basis of the numerous experimental data available:

1. With the occasional exception of magnesium salts, a direct toxic effect of a particular salt in a mixture of salts rarely constitutes an obvious major problem, owing to the fact that the various ions in the mixture mutually counteract each other's toxicity (antagonism).

2. The factor limiting the yield is the total concentration of salts, which may affect the plant directly (over-absorption of salts) and indirectly (shortage of water available for uptake in the roots).

In this country the problems concerning salt injuries are found almost exclusively in connection with intensive crop production, *e.g.*, in green house cultures in which the plants need sizable and frequent supplies of nitrate, and in which they are also watered frequently. In this case the nitrate content of the soil fluctuates constantly between a harmful excess of nitrate ions in the soil water (as shown by increasing conductivity values), and a crop reducing deficiency caused by leaching. Under circumstances such as these a more even supply of nitrate might be desirable. In this connection the idea was conceived of using an anion exchange resin, which as a means of supplying nitrate to the plants offers a number of advantages: 1) It is possible in this way to supply additional nitrogen without increasing the ion

concentration of the soil water. 2) The procedure makes it possible to water the plants vigorously without giving rise to leaching of nitrate from the top soil. 3) Further, the nitrate uptake of the plants will take place more continuously, *viz.*, *pari passu* with root respiration (bicarbonate ions from the respiratory process liberate nitrate ions from the exchange resin).

Method

The experiments were carried out in 15 litre culture pots according to the following scheme. To the control series (the c-series) calcium nitrate ($\text{Ca}(\text{NO}_3)_2$) was supplied in amounts of, respectively, zero, 0.5, 1, 2, 4, 6, 8, and 10 g. of N per pot, while nitrate on the exchange resin in the x-series was supplied in amounts of, respectively, 0.5, 1, 2, 4, 6, and 8 g. of N per pot. Each concentration step was run in triplicate. After being counterpoised by means of granite chips each vessel was filled with 18.0 kg. of sandy mould from Hornum Experimental Station. After addition of basic fertilizers the following analytic values was found: pH 7.3, Phosphate Value 7.8 (mg. of PO_4 dissolved when 10 g. soil was shaken for 3 hours with 250 ml. of 0.2 N sulphuric acid), Potash Value 16.7 (kiloequivalents of exchangeable potassium per 2.5 kg. of soil), and Soil Extract Conductivity Value 1.75 (specific conductivity expressed in 10 times millimhos/cm. at 20°C of a 1 : 22.5 soil : water extraction).

The crop used was Yellow Mustard (*Sinapis alba*) sown at a density of 30 seeds per pot. Through daily control weighings and addition of distilled water the water content of the pots was kept at a steady 60 per cent of the water capacity of the soil. The additional water was supplied to the from pots below through a side tube, thus eliminating the possibility of washing out nitrate from the control series.

The exchange material used was Dowex 2, a strong anion exchange resin. It was purchased in the chloride form with a total capacity of 3.2 kiloequivalents of anion per g. of dry exchange resin. The resin was washed a couple of times with a 10 per cent solution of HCl, and subsequently with distilled water, and was then transferred to a column through which an 8 per cent solution of HNO_3 was slowly passing. This process was continued until chloride ions were no longer present in the eluate. Finally the exchange resin was washed with distilled water to neutral reaction, and was then ready for mixing with the soil.

Table 1. Conductivity values at the beginning of the experiment.
c without, x with NO_3 on ion exchange resin.

N added per vessel (g.)	c-series	x-series
0	1.75	1.75
0.5	1.81	1.67
1	2.03	1.64
2	2.46	1.78
4	3.25	1.69
6	4.33	1.54
8	5.48	1.72
10	6.53	—

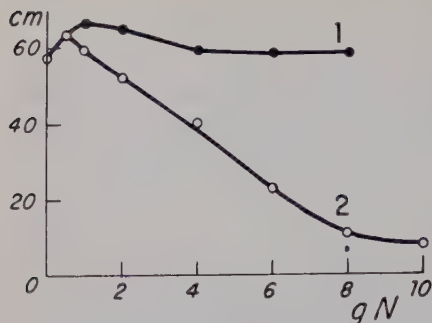


Figure 1. Height of plants as a function of N added (g. per vessel). 1. NO_3 via ion exchange resin, 2. the control series.

Table 1 shows that resin-bound nitrate added to the pots does not increase the conductivity value of the soil. Whereas a concentration of 8 g. of N per pot in the control series increased the conductivity value of the soil from 1.75 to 5.48, the same amount on the ion exchanger produced no increase in the conductivity value as compared to the pots without any nitrate addition.

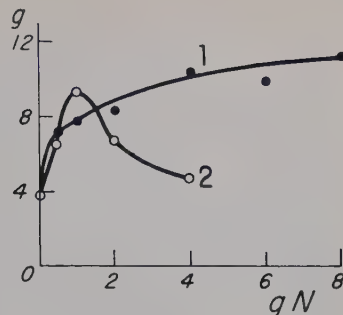
The sowing was done on May 28th, 1958. After thinning to 16 plants per pot on June 19th, 6 uniform plants without roots were taken for analysis from each pot on July 3rd. No samples were taken from the three pots most heavily fertilized in the control series, where growth was defective due to salt injury. The month of July having been very rainy, the pollination of the remaining plants was very uneven, and hence only the yield and the analyses resulting from the July 3rd samples representing the maximum vegetative development are included in the present paper.

Results

Figure 1 shows the height of the plants as a function of the nitrogen added. By the middle of June the plants given only basic fertilizers (no addition of N) were the tallest, but by the 3rd of July they were found to be lower than the plants in the pots containing 0.5 g. of N , due to nitrogen deficiency. The height of the plants in the control series decreases nearly linearly with the addition of nitrate (and hence with the concentration of salts), the curve flattening out somewhat for 8 and 10 g. of nitrogen added, however. The plants in the resin pots of the x-series are of almost equal height regardless of the amount of nitrate, but there does appear to be a slight depressive effect for the three highest concentrations.

The yield of dry matter as a function of the amount of nitrogen added to the pots appears in Figure 2. While the yield in the control series reaches a maximum value of 9.3 g. for the addition of 1 g. of nitrogen and then rapidly decreases according to a concave curve, a steadily increasing yield is obtained in the anion exchange pots with a maximum value of 11.1 g. for 8 g. of N added.

Figure 2. Yield (g. dry matter per plant) as a function of N added (g. per vessel). 1. NO_3 via ion exchange resin, 2. the control series.



The dry matter content of plants from resin pots was found to be somewhat higher than that of plants from the corresponding control pots (Table 2).

Table 2 and Figure 3 show the interrelationship between the amounts of nitrogen added and taken up. While the uptake from the only slightly N-fertilized control pots to some extent exceeds that from the corresponding ones in the x-series, the reverse is the case when the amount of nitrogen added per pot increases to a value of 4 g. Whereas in the control pots the uptake decreases strongly with additions of nitrogen higher than 2 g., there appears to be a steady increase in the resin pots of the x-series.

The yield of dry matter as a function of the amount of nitrogen taken up is shown in Figure 4. Up until an amount of 300 mg. of nitrogen taken up per plant the two curves are nearly identical, indicating that the nitrogen taken up is utilized with equal efficiency whether it originates from the nitrate ions of the soil water or from the nitrate supplied by ion exchange. Above this value an increased uptake causes an increase in yield in the resin pots, whereas in the control pots an increased uptake results first in a decreasing yield, and in the case of the pot most heavily fed (4 g. of N) the salt injury effect is so strong as to cause a reduction of both uptake and yield, i.e., the curve turns back towards the origin of the coordinate system.

Table 2. Dry matter content and amounts taken up of N, K, Ca, Mg, and P in mg. per plant. c without, x with NO_3 supply via ion exchange resin.

N added per vessel (g.)	Dry matter		N		K		Ca		Mg		P	
	c	x	c	x	c	x	c	x	c	x	c	x
0	17.35	17.35	105	105	123	123	58	58	4.6	4.6	2.4	2.4
0.5	15.51	16.01	189	173	255	256	81	88	6.3	8.5	4.0	2.6
1	13.30	14.09	281	212	440	343	136	93	10.9	5.9	3.6	2.9
2	11.19	13.00	327	299	392	394	140	123	12.1	10.5	2.7	3.2
4	11.65	12.63	208	373	267	488	114	166	6.3	11.4	1.7	3.8
6	—	12.21	—	395	—	474	—	162	—	13.8	—	3.6
8	—	12.31	—	440	—	501	—	161	—	14.2	—	4.5

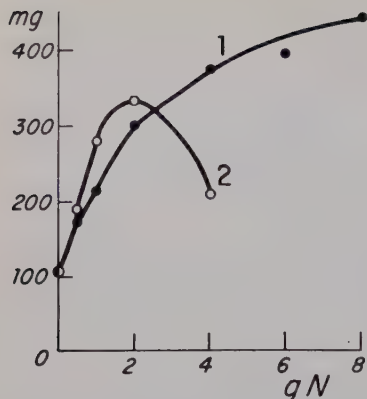


Figure 3. *N* taken up (mg. per plant) as a function of *N* added (g. per vessel). 1. NO_3 via ion exchange resin, 2. the control series.

In other words: 1) A beginning reduction of the yield due to a too high concentration of salts is caused primarily by a less efficient utilization during the production of matter of the nitrogen taken up. 2) At higher concentrations of nitrate the reduction in yield may possibly be due to a decreased uptake of nitrogen as well as to a less efficient utilization of the nitrogen taken up.

For the less heavily fertilized control pots the uptake of the nutrients K, Ca, Mg, and P (Table 2) in certain cases exceeds slightly that in the corresponding pots of the x-series, whereas in the most strongly fertilized pots without anion exchange resin the uptake of these nutrients — as in the case of N — is inhibited to a very considerable extent.

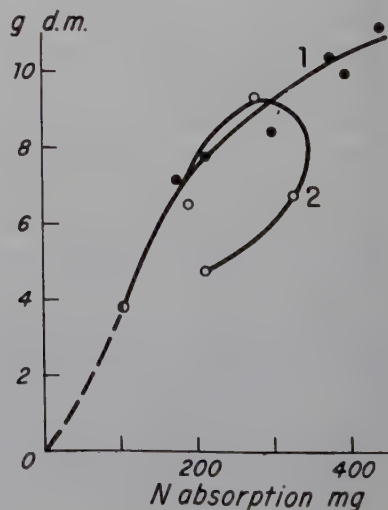


Figure 4. Yield (g. dry matter per plant) as a function of *N* taken up (mg. per plant). 1. NO_3 via ion exchange resin, 2. the control series.

Table 3. *Relative contents of N, K, Ca, Mg, and P in the dry matter.*
c without, x with NO₃ supply via ion exchange resin.

N added per vessel (g.)	% N		% K		% Ca		% Mg		% P	
	c	x	c	x	c	x	c	x	c	x
0	2.75	2.75	3.23	3.23	1.52	1.52	0.121	0.121	0.062	0.062
0.5	2.91	2.45	3.93	3.63	1.25	1.25	0.097	0.121	0.061	0.037
1	3.02	2.75	4.73	4.44	1.46	1.20	0.117	0.076	0.039	0.037
2	4.88	3.59	5.85	4.73	2.09	1.48	0.181	0.126	0.041	0.039
4	4.46	3.63	5.73	4.75	2.45	1.61	0.136	0.111	0.037	0.037
6	—	4.02	—	4.83	—	1.65	—	0.141	—	0.037
8	—	3.97	—	4.52	—	1.45	—	0.128	—	0.041

The relative contents of N, K, Mg, and P in the dry matter appear from Table 3. The Table shows the contents of N and K to increase rather considerably with increasing supplies of nitrogen. The increases in Ca and Mg are less pronounced, whereas the P content on the whole remains unchanged with increasing amounts of N in the pots. In nearly all cases the concentrations are considerably higher in plants from control pots than in plants from ion exchange pots. Hence the ash contents of the dry matter of the latter plants becomes the lower one, and consequently their content of organic matter is higher than that of the plants from pots without resin.

Discussion

Steenbjerg (1946) showed that a salt injury effect usually becomes appreciable at a conductivity value of 3. A comparison of Figure 2 with Table 1 shows the salt injury effect in control pots of the present experiment to become appreciable already at a conductivity value 2 to 2.5, and a value of 3.25 is seen to have caused a reduction of 50 per cent in the yield. However, studies by other authors suggest that it is not possible to establish a general limit for the conductivity value causing reduction of the yield, as Richards (1954) finds considerable differences in the salt tolerance of various species of plants, as well as variations in the salt tolerance of each individual plant according to its stage of development.

Wadleigh and Ayers (1954) found a high degree of correlation between the yield of beans and the diffusion pressure deficit of the soil liquid, regardless of whether the latter is caused by a reduction of the water content of the soil, or by the addition of increasing amounts of NaCl. The interrelationship between the concentration of salt and the yield found by Wadleigh and Ayers is in good agreement with the curve for the control pots presented in Figure 2.

The results of the control series pretty well confirms the latter of the conclusions drawn by Grillo (see the introduction). Figure 2 in connection with Table 1 is showing the total concentration of salts to be the factor limiting the yield. But it appears to be difficult on the basis of the present experimental results to determine to what extent the reduction in yield is due to an increased uptake of salts, or to what extent it is caused by a reduction in availability of the water. Table 3 shows that, N, K, and Ca in particular are found at much higher concentrations in the plants in the most heavily fertilized control pots than in those from the corresponding and even more heavily fertilized resin pots. The reason for this could perhaps be that the plants from the control series have suffered an increased loss of matter due to a higher respiratory rate during the increased uptake of ions and to the maintenance of the steeper concentration gradient.

The less efficient utilization of the nitrogen taken up during the production of matter in plants from heavily fertilized control pots, as appears from Figure 4, may possibly be due to a reduction in the rate of photosynthesis via closing of the stomata as caused by a decreased availability of the water. This hypothesis agrees with the observation made by Schneider and Childers (1941), *viz.*, that the rate of photosynthesis is reduced, and that of respiration is increased, in plants growing in soil, the water content of which is not at its optimum value.

The main conclusion to be drawn from the data obtained in the present study is that it appears to be possible to avoid salt injury effects and to obtain a better yield of the nitrate supplied by adding it bound to a resin instead of employing the usual fertilizer procedure. There is no doubt that the ion exchange method of supplying nitrate as fertilizer can be easily practiced also on a larger scale, whether or not this will actually be the case, and when, appears to be a purely financial question, as the ion exchange resins are so far rather costly.

Summary

In pot experiments with Yellow Mustard a comparison is made of 1) addition of increasing amounts of nitrate to soil in the usual way and 2) addition of similar amounts of nitrate ions bound to an anion exchange resin. The data show that in the latter case it is possible to add large amounts of nitrate without increasing the conductivity value of the soil, and without causing salt injuries to the crop in question.

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Growth Response of Pea Roots to 2,4-D Applied to the Hypocotyl

By

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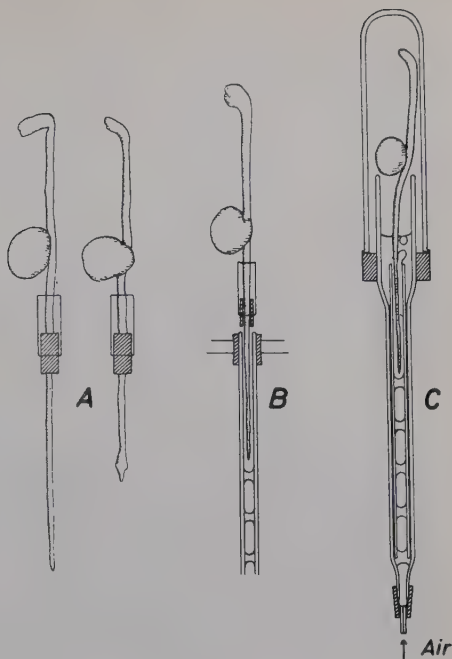
From experiments with labeled 2,4-dichlorophenoxyacetic acid (2,4-D) it is known that this substance when applied to the aerial parts of the plant may be translocated down into the roots (Crafts 1956, Fang 1958). In this paper experiments regarding the growth response of roots of pea seedlings to 2,4-D applied to the hypocotyl are reported. When the roots are grown on moist filter paper even low doses of 2,4-D applied in this way cause growth inhibition. A comparison of this response with the response of the roots to 2,4-D in the growth solution gives some interesting indications regarding the complexity of the 2,4-D action on the growth processes in pea roots.

Methods and Results

Application of 2,4-D to the Hypocotyl

Pea seeds (*Pisum sativum*) were germinated on moist filter paper for 2 days at 22°C. Plants with a root length of about 2 cm. were selected for the experiment. A 15 mm. long glass tube with an inner diameter of 3 mm. was fastened around the hypocotyl with a piece of rubber tubing as shown in Figure 1 A. In order to prevent leakage of the solution from the glass tube, the tubing was dipped into melted lanolin before use. For the further growing of the pea seedlings polyethylene sacks (23×15 cm.) fastened on masonite disks and fitted with double layers of filter paper were used. In the upper parts of each sack 10 seedlings were placed in a row and fixed with a glass rod which was pressed against the tubes around the hypocotyl. The sacks were kept in a position only slightly deviating from the vertical during the whole growth period. For moistening the filter paper 50 ml. nutrient solution of the composition given by Eliasson (1958) were supplied to each sack. The positions of

Figure 1. A. *Pea seedlings with glass tubes for application of 2,4-D solution to the hypocotyl.* The plant to the right shows the typical appearance of the root 24 hours after supply of 0.1 μg . 2,4-D in 0.02 ml. buffer solution with pH 3.5 in the tube. B. *Device used for study of root elongation in flowing solution when 2,4-D is applied to the hypocotyl.* Fresh solution and air is continuously streaming upwards through the lower glass tube. C. *Device for following root growth in a small amount of circulating solution.*



the root tips were marked off on the polyethylene sack. The growth in length of the roots was measured from this mark at the times desired. Before application of the growth substance the roots were left to grow for a certain period, usually 18 to 20 hours. The growth substance was dissolved in citrate-phosphate buffer ($2 \times 10^{-3} M$ citric acid, $10^{-3} M K_2HPO_4$, pH 3.5). Of this solution 0.02 ml. was pipetted into the glass tube surrounding the hypocotyl. To the control 0.02 ml. buffer solution was applied. This method will in the following be referred to as the root translocation (RT) method.

The curves of Figure 2 show the effect on root elongation of the application of 0.1 and 1 μg . 2,4-D to each plant in the manner described. After application of 0.1 μg . 2,4-D the roots grow with an unchanged rate for 4 to 5 hours. After this time some roots stop elongation completely while other continue to grow but at a reduced rate. A few millimeters above the tip typical swellings appeared during the first 24 hours after treatment (Figure 1 A). Later the tip of the main root was further deformed. There also occurred a vigorous formation of lateral roots, which after three days had reached a length of about 5 mm. The growth of the epicotyl was not influenced by 0.1 μg . 2,4-D. To 2,4-D quantities lower than 0.1 μg . the response is rather varying, but a considerable fraction of the plant material used responded with an inhibition of root elongation even to 0.02 μg . 2,4-D per plant. At this low 2,4-D dose, however, most of the inhibited roots showed recovery

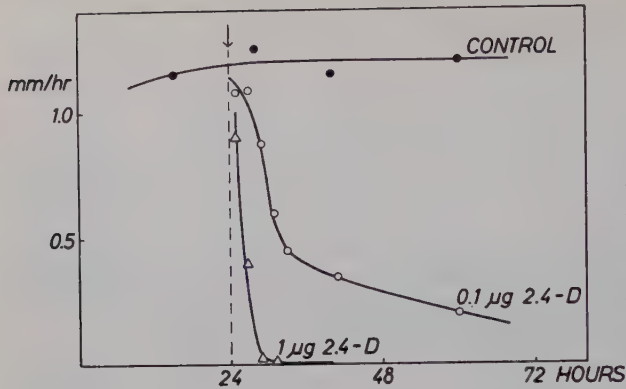
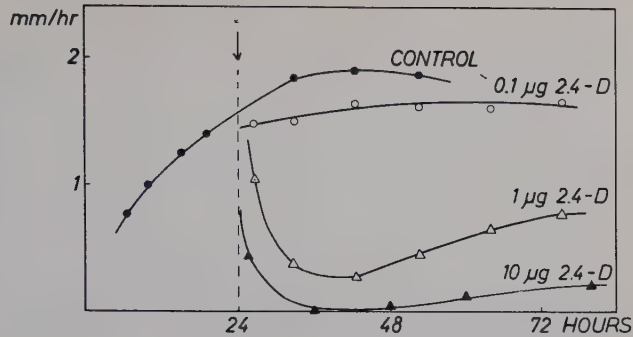


Figure 2. Effect on root elongation of 2,4-D application to the hypocotyl of pea seedlings growing on filter paper. In the experiment 10 plants have been used for each treatment. 2,4-D was supplied at the time indicated by the arrow.

of growth after a day or two. When the 2,4-D dose used was 1 μg . per plant elongation of the roots was completely inhibited after 2 or 3 hours and later there was no further elongation of the main root. The root swellings of the type shown in Figure 1 A occur also in this case, but they are somewhat more delayed and followed by swelling of the whole root in connection with formation of numerous initials of lateral roots. After a few days the roots were deformed in much the same way as described for bean roots by Wilde (1949). The growth of the epicotyl was decreased by this 2,4-D dose. For 10 μg . 2,4-D responses of the roots were largely the same as for 1 μg ., but the growth in length of the epicotyl showed stronger inhibition and also swelling.

It was noticed that roots growing in close contact with the filter paper showed a weaker response to 2,4-D application than roots with poor contact with the filter paper. A probable explanation of this observation would be that a part of the translocated 2,4-D leaks out from the root and is transferred to the filter paper. In order to investigate this matter some experiments were carried out with the device shown diagrammatically in Figure 1 B. The seedlings were fitted with glass tubes for application of 2,4-D solution to the hypocotyl, and the roots were grown in narrow glass tubes with continuously flowing solution according to a method described earlier (Eliasson 1958), in the following called the CF method. These experiments showed that the roots are not so sensitive to 2,4-D application to the hypocotyl when they are continuously washed with fresh solution as when growing on filter paper. Not even 10 μg . produces complete inhibition of root elongation, and the inhibition is to a certain degree reversible. The results of one of the experiments are given in Figure 3. These results may be explained with the assumption that 2,4-D leaks out from the roots and is removed by the solution when the roots are grown according to the CF method. Earlier experiments with wheat seedlings suggest that exuded substances are effectively removed from

Figure 3. Root elongation in continuously renewed solution after application of 2,4-D to the hypocotyl. For this experiment the device shown in Figure 1 B was used. Each treatment comprised 10 plants.



the root surface with this method (Eliasson 1959). If secondary substances formed under the influence of 2,4-D are involved in the growth inhibition also leakage and removal of such substances may contribute to the divergence in 2,4-D effect between roots grown on filter paper and in CF tubes. The 2,4-D inhibited roots swell somewhat even when grown in CF tubes but not to the extent obtained on filter paper. A conspicuous difference is that root hairs, which develop richly on roots grown on filter paper especially after 2,4-D treatment, are lacking on roots grown in the CF tubes.

Effect of 2,4-D in the Growth Solution on Elongation of Pea Roots

In order to provide a comparison to the RT experiments also the effects on root growth of 2,4-D solutions brought in contact with the whole of the roots were investigated. The composition of the nutrient solution in which 2,4-D was dissolved was the same as that used in the RT experiments. Both the effect of a large amount of solution which was continuously renewed and the effect of a small amount of solution which was not renewed was investigated. In the experiments of the former type the CF technique (cf. above) was employed. About 100 ml. solution were used for each plant per day. Time curves for the rate of root elongation in various 2,4-D concentrations obtained with this method are given in Figure 4. Figure 5 shows the result of a more detailed experiment on the effect of 2,4-D supplied to the roots at different ages.

The device used for the experiments with a small amount of solution is shown in outline in Figure 1 C. About 1 ml. solution was circulated by an air stream and passed the root in the inner tube together with the air. The main difference in growth conditions between this method and the CF method is thus that the solution is not renewed. The amount of solution which evaporated (0.1 to 0.3 ml. per day) was compensated for by adding new

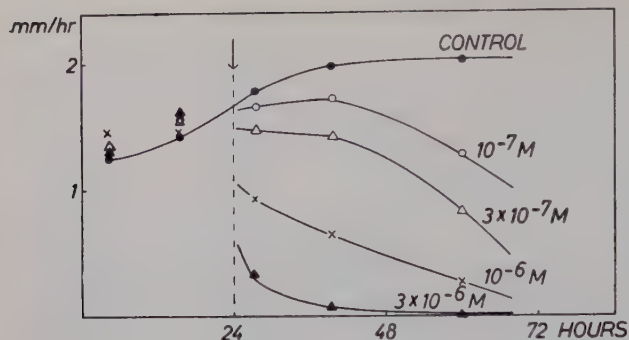


Figure 4. Root elongation in continuously renewed solution with 2,4-D supplied to this solution. The roots were grown 24 hours in the control solution before changing to the 2,4-D containing solutions. The figures give the concentration of 2,4-D used. Each curve was obtained as an average of 24 roots.

solution. The surprising result obtained with this method is that the sensitivity to the inhibitory effect of 2,4-D is greater if the roots are grown in a small amount of a solution with a certain 2,4-D concentration than if the solution containing 2,4-D is continuously renewed. In Figure 6 the growth curves obtained in an experiment with 3×10^{-7} M 2,4-D are given. These curves show the average growth of 12 roots. The response of the individual roots, however, differed from the time course shown by the curves and was characterized by a sudden stop in the elongation a certain time after 2,4-D addition. The time elapsed before this growth cessation occurs varies greatly between the individual plants and shows also some dependence on the age of the root. Of the 12 roots supplied with 2,4-D 48 hours after the beginning of the experiment of Figure 6, 3 had stopped growing after 7 hours, 8 after 13 hours and after 24 hours only one root was still elongating. The roots to which 2,4-D was added 24 hours earlier ceased to grow around 24 hours after 2,4-D addition. The most likely explanation to this cessation of growth

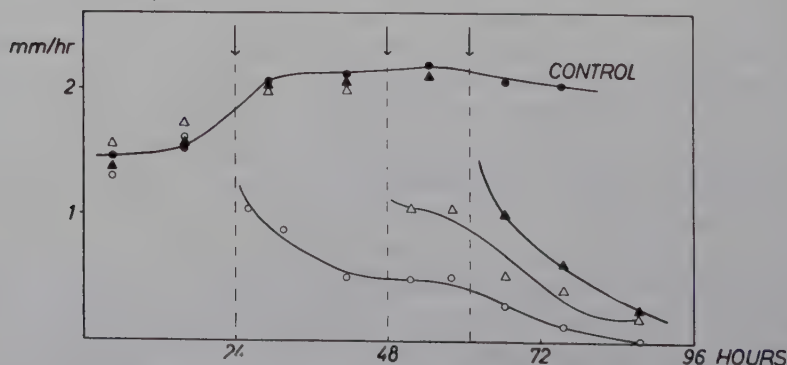
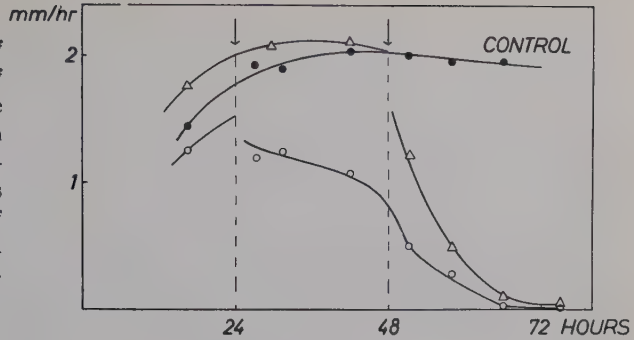


Figure 5. Effect of 10^{-6} M 2,4-D supplied in flowing solution on root elongation. The arrows indicate changing to 2,4-D solution. 12 plants were used for each treatment.

Figure 6. Growth in length of pea roots in a small amount of circulating solution. The device in Figure 1 C was used. Each plant was grown in 1 ml. solution. To this solution 2,4-D was added to a concentration of 3×10^{-7} M at the times indicated by the arrows. For each treatment 12 roots were used.



seems to be that the roots in the presence of 2,4-D exude some inhibitory substance which after reaching a certain concentration stops the root growth. After the cessation of growth the root tips rapidly developed swellings of the same type as those obtained with the RT method (Figure 1 A).

The growth rate of the controls was considerably higher when the roots were grown in tubes than when they were grown on filter paper. This was also the case for roots fitted with tubes around the hypocotyl (Figure 3). The divergence may be due to a more abundant water supply or to a more effective removal of natural growth inhibitors in the tubes (cf. Eliasson 1959).

Discussion

Application of 2,4-D to the hypocotyl of pea seedlings according to the RT method gives a series of growth responses which depend on the amount of 2,4-D. These responses seem to be rather specific for 2,4-D and related synthetic growth regulators. In preliminary experiments about the same responses were obtained with 2-methyl-4-chlorophenoxy-acetic acid as with 2,4-D. Indolyl-3-acetic acid had no effect when applied in doses lower than 1 μ g. per plant and 10 μ g. per plant caused only a temporary inhibition of root elongation. Although as yet the experience of the method is not very extensive, some preliminary experiments indicate that the RT method is useful as an assay method for 2,4-D in plant extracts.

The main purpose of the present experiments was, however, to investigate the usefulness of growth responses of roots as an indication of translocation of growth substances into roots in analogy with the use of growth responses of bean stems and leaves for study of translocation of 2,4-D in aerial parts of bean plants (Mitchell and Brown 1946, Rice 1948, Day 1952). Although the experiments have been successful to the extent that a satisfactory growth

response was obtained in the roots, they also point to some difficulties connected with such a technique. One of these difficulties is that the degree of contact between the growth solution and the roots affects the response. This fact is most simply explained by the assumption that 2,4-D leaks out from the roots into the surrounding solution (cf. Clor and Crafts 1957). The interpretation of the experiments is, however, complicated by the evidence that a leakable substance formed in the roots in the presence of 2,4-D may be partly responsible for the growth inhibition. It is possible that this substance may be formed in the mature parts of the roots and translocated to the growth zones thus giving a growth inhibition independent of translocated 2,4-D.

Summary

A technique for the application of small amounts of solution containing growth substances to the hypocotyl of pea seedlings is described. Small doses of 2,4-D applied in this way cause inhibition of root elongation and swellings of the roots. Washing of the roots counteracts the effect of 2,4-D.

The growth response of pea roots to 2,4-D applied in continuously renewed solution and in a small amount of circulating solution has also been investigated. The sensitivity of the roots to a given concentration of 2,4-D was found to be greater in the circulating solution. This fact is taken as an indication that a leakable, growth-inhibitory substance is formed in the roots in the presence of 2,4-D.

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Zur Beeinflussung der morphoregulatorischen Wirksamkeit von 2,3,5-Trijodbenzoesäure (TIBA) durch α -Naphthylessigsäure

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Einleitung

Gorter gelang es 1951 an Tomaten-Pflanzen durch Zuführen von Hemmstoff (2,3,5-Trijodbenzoesäure; TIBA) ringfaszierte Sprosse zu induzieren. Auffallende teratologische Phänomene konnten van Zeist und Koevoets (1951) und Harder und Oppermann (1952) mit TIBA bei *Kalanchoe blossfeldiana* hervorrufen, während Steveninck (1956) über Ringfasziation durch 2,4-Dichlorphenoxyessigsäure bei *Lupinus luteus* berichtete.

Wie Wenck (1952) bei Versuchen an *Codiaeum variegatum* f. *interruptum* zeigen konnte, war es dort möglich den teratogenen Effekt der 2,3,5-Trijodbenzoesäure durch gleichzeitiges Zuführen von Wuchsstoff (in ihren Versuchen Indol-3-essigsäure und 2,4-Dichlorphenoxyessigsäure) zu kompensieren. Da bezüglich der Zellstreckung vielfach eine gegenseitige Wirksamkeitsbeeinflussung von TIBA und Wuchsstoffen beobachtet wurde (Galston 1947, Thimann und Bonner 1948, Linser 1954) scheint die Frage, ob auch die morphoregulatorische Wirkung (insbesondere die TIBA-induzierte Ringfasziation) bei *Lycopersicon esculentum* wie sie von Gorter beschrieben wurde durch gleichzeitiges Zuführen von Wuchsstoff beeinflusst werden kann von besonderem Interesse. Zur Untersuchung dieser Frage wurden die nachfolgend dargelegten Versuche durchgeführt.

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Material und Methodik

Als Versuchsmaterial kamen Keimpflänzchen von *Lycopersicon esculentum* (Sorte „Brunner Beste“) zur Verwendung. Die Samen wurden am 16. März 1959 in kleine Holzkisten in Komposterde gesät. Zehn Tage später waren Keimpflanzen entwickelt, an welchen 2 große Cotyledonen bereits entfaltet und das erste Blättchen schon deutlich erkennbar war. Die Blattlängen betrugen zu diesem Zeitpunkt (Mittelwerte aus drei Messungen):

Cotyledonen = 20,3 mm, 1. Blatt = 2,6 mm, 2. Blatt = 1,5 mm, 3. Blatt = 0,4 mm, 4. Blatt = 0,1 mm.

Die Längenbestimmungen erfolgten mit Hilfe eines Okular-Meßplättchens unter dem Stereo-Mikroskop. Nach dem 4. Blatt, das als kleine Anlage am Apex erkennbar war, folgte stets der Vegetationskegel.

Von jeweils 5 Pflanzen des beschriebenen Stadiums wurden die Wurzeln mit Filterpapier, das stark mit Aqua dest. befeuchtet war, umwickelt. Danach wurden die auf diese Weise zu 5 gebündelten Pflanzen mit dem Apex nach unten in die Versuchslösung eingetaucht, ohne daß das Filterpapier mit der Versuchslösung in Berührung kam. Nach einer Tauchzeit von je 15 Minuten wurden die Pflänzchen leicht mit Filterpapier abgetrocknet und danach reihenweise in Holzkistchen in Komposterde pikiert und im Glashaus bei normalen frühlingsmäßigen Lichtverhältnissen aufgestellt.

An Versuchslösungen kamen wässrige Lösungen von 2,3,5-Trijodbenzoesäure (TIBA) und α -Naphthylelessigsäure (NAA) in Konzentrationen von 10^{-1} , 10^{-2} und 10^{-3} ‰ zur Verwendung. Bei den Versuchen mit Mischungen von TIBA mit NAA wurden jeweils 2×10^{-1} , 2×10^{-2} und 2×10^{-3} ‰ ige Stammlösungen von TIBA im Verhältnis 1 : 1 mit gleichkonzentrierten Stammlösungen von NAA entsprechend gemischt.

Pro Konzentration bzw. Mischung kamen jeweils 5 Pflanzen zur Verwendung. Als Kontrolle wurden 5 Pflanzen mit Aqua dest. behandelt.

Die Auswertung der Versuche wurde am 11.5.1959, also 48 Tage nach der Wirkstoffapplikation durchgeführt. Von jeder Probe wurde dabei bestimmt:

1. Die Gesamtlänge der Pflanzen (oberirdischer Teil bis zum Apex);
2. die Blattanzahl des Haupttriebes bis zur Blüte;
3. die Anzahl der mit freiem Auge noch sichtbaren Blüten pro Infloreszens des Haupttriebes;
4. der morphologische Habitus der Pflanzen;
5. die minimalen und maximalen Längen der Seitentriebe.

Den hier veröffentlichten Versuchen gingen mehrere orientierende Versuche voraus, welche hier nicht weiter beschrieben werden sollen, die jedoch im wesentlichen gleiche Ergebnisse brachten.

Experimenteller Teil

a) Die Wirkung von TIBA in verschiedener Konzentration

Achtundvierzig Tage nach der Wirkstoffbehandlung zeigten sich an den mit stärkeren TIBA-Konzentrationen behandelten Versuchspflanzen gegenüber den Kontrollen starke morphologische Unterschiede. Während die un-



Abb. 1. *Lycopersicon esculentum* („Bonner Beste“): Haupttrieb 23 Tage nach der Behandlung mit 10^{-2} % TIBA; links: Ringfasziiertes Apex mit beginnender Differenzierung der Blütenprimordien (Vergr. ca. 5 mal); rechts: Ringfasziiertes Apex von oben fotografiert mit Blütenprimordien an welchen die Anlagen der Kelchblätter deutlich erkennbar sind.

behandelten Kontrollpflanzen zu diesem Zeitpunkt eine Höhe von 40 mm erreicht und normal hellgrüne Blätter entwickelt hatten, waren die mit 10^{-1} % TIBA behandelten Pflanzen stark in ihrem Gesamtwuchs gehemmt und zeigten verschmälerte tief dunkelgrün gefärbte Blätter. Die Vegetationspunkte des Hauptsprosses, aber auch die meisten Apices der Seitentriebe, zeigten alle die von Gorter (1951) beschriebene Ringfasziation (Abb. 1). Auffallend war, daß der ringfasziierte Apex, auf welchem sich stets eine große Anzahl Blütenanlagen entwickelten (Abb. 1 rechts) schon nach 4,4 Blättern (Mittelwert aus 5 Zählungen) in Erscheinung trat, wogegen bei den Kontrollpflanzen normale Infloreszenzen erst nach dem 7,8. Blatt des Hauptsprosses erschienen. Die Wirkstoffzufuhr hatte demnach einen beschleunigenden Einfluß auf die Ausbildung von Blüten (vergl. Waard und Roodenburg, 1948). Der Einfluß anderer Konzentrationen sowie der von NAA und der Mischung von TIBA und NAA auf die Blütenbildung ist aus den Abb. 2 und 3 ersichtlich. Aus den Cotyledonarseitentrieben entwickelten sich ebenfalls ringfasziierte, später mit Blütenanlagen besetzte Vegetationspunkte, die entweder ohne oder aber mit ein bis zwei Vorblättern erschienen. Die Entwicklung der Seitentriebe war durch die starke TIBA-Konzentration stets gefördert, wobei sich das erste Blättchen der Cotyledonartriebe vielfach ungefiedert

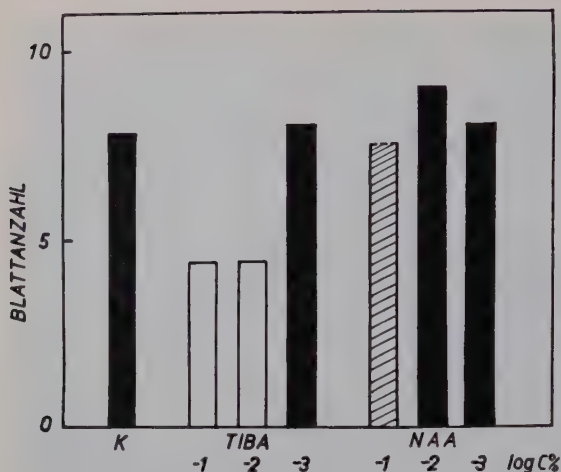


Abb. 2. *Lycopersicon esculentum* („Bonner Beste“): Blattanzahl bis zur Infloreszenz (oder Ringfasziation) nach Behandlung mit TIBA bzw. NAA. Schwarze Säulen: \pm normale Blütenstände, weiße Säulen: ringfaszierter Apex mit Blüten, schraffierte Säulen: vegetative Pflanzen, Blattanzahl zum Zeitpunkt der Untersuchung.

zeigte. Auch an den Blättern des Hauptsprosses waren formative Veränderungen zu erkennen, welche darin bestanden, daß mit zunehmender Blattzahl allgemein eine Verringerung der Fiederanzahl zu beobachten war. (Vergl. Laibach und Mai 1936; Linser et al. 1955 und Hanf 1957). Nach drei bis vier solcherart veränderter Blätter trat meistens wieder eine Normalisierung der Blattform ein.

Bei der schwächeren TIBA-Konzentration von 10^{-2} ‰ waren die Pflanzen weniger in ihrem Gesamtwuchs gehemmt. Die Gesamtlänge des Hauptsprosses betrug hier 96 mm (Mittelwert aus 5 Messungen) gegenüber 116 mm der Kontrolle. (Vergl. Abb. 4, auf welcher auch der Einfluß anderer Konzentrationen von TIBA, sowie der von NAA und der Mischung TIBA/NAA auf die Gesamtlänge der Versuchspflanzen ersichtlich ist). Ringfasziation konnte auch bei dieser Konzentration stets beobachtet werden. Dabei fiel jedoch auf, daß vielfach die Apices nicht vollständig zu Ringen verwachsen waren (wie dies beispielsweise bei dem auf Abb. 1 dargestellten Apex der Fall war), sondern mannigfaltig geformte Gebilde, welche als Teilstücke von Ringen aufgefaßt werden müssen, zu beobachten waren. Offensichtlich reichte hier die schwächere TIBA-Konzentration nicht mehr aus um vollständig faszierte Ringe zu induzieren. Auch an den Blütenanlagen und den sich daraus entwickelnden Blüten waren mannigfaltige teratologische Veränderungen zu beobachten, von denen insbesondere die Bildung von „Riesenblüten“ mit einer enormen Erhöhung aller Blütenelemente (z.B. bis über 50 Kelchblätter) auffiel.

So wie bei der starken Konzentration waren auch hier die ringfaszierten

Tabelle 1. Die Wirkung verschiedener Konzentrationen von TIBA bzw. NAA auf verschiedene morphologische Größen bei *Lycopersicon esculentum* („Bonner Beste“). Die Zahlen stellen Mittelwerte aus je 5 Versuchspflanzen dar. K Kontrolle; N Normal; RF Ringfasziation; + leicht, ++ mäßig, +++ stark morphologisch verändert.

Substanz	Log. C %	Gesamtlänge (mm)	Blatthabitus	Länge der Cotyledonartriebe (mm)	Blattanzahl ohne Cotyledonen bis zur Blüte	Ringfasziation	Bemerkungen
K	—	116	N	35	7,8	—	
TIBA.	—1	66	+++	30	4,4	RF	
TIBA.	—2	96	++	100	4,4	RF	
TIBA.	—3	159	N	65	8,0	—	
NAA.....	—1	22	N	—	7,5	—	vegetativ
NAA.....	—2	91	N	25	9,0	—	
NAA.....	—3	144	N	35	8,0	—	

Apices und die sich darauf entwickelnden Blüten schon nach 4,4 Blättern des Haupttriebes zu beobachten.

Die Entwicklung der Seitentriebe war hier gegenüber der höheren Konzentration noch stärker gefördert (vergl. Tab. 1) was in der Folge dazu führte, daß der ohnedies gehemmte Haupttrieb von den Seitentrieben, insbesondere den Cotyledonartrieben an Länge und Stärke weit übertroffen wurde. Die Infloreszenzen dieser Seitentriebe waren normal entwickelt und Ringfasziation war nirgends zu beobachten. Die schwächere Wirkstoffkonzentration dürfte hier nicht mehr ausgereicht haben, auch in den Seitentrieben einen teratogenen Effekt zu entfalten. Während die Blätter des Hauptsprosses noch deutliche morphologische Veränderungen (Blattverschmälerungen, Verringerung der Fiederanzahl) zeigten und außerdem dunkelgrün erschienen, waren die Blätter der Seitentriebe meist normal.

Bei der schwächsten in unseren Versuchen verwendeten TIBA-Konzentration von 10^{-3} % traten an den Versuchspflanzen keine morphologischen Veränderungen auf. Auffallend war, daß hier TIBA auf die Gesamtlänge des Hauptsprosses einen fördernden Einfluß ausübte, zumal diese im Mittel bei den behandelten Pflanzen um 43 mm größer war als bei den Kontrollpflanzen. Obwohl in keinem Falle Ringfasziation beobachtet werden konnte, waren die Endblüten der Infloreszenz des Haupttriebes häufig zu „Riesenblüten“ mit einer gesteigerten Anzahl aller Blütenelemente und zum Teil stark faszierten Blütenstielen entwickelt. Zur teratogenen Ausbildung der Endblüten genügte offenbar schon eine sehr geringe TIBA-Konzentration. Eine Förderung der Blütenbildung gegenüber den Kontrollen war durch TIBA 10^{-3} % nicht zu beobachten. Die Infloreszenzen erschienen nach dem 8. Blatt des Hauptsprosses (vergl. Abb. 2).

b) Die Wirkung von NAA in verschiedener Konzentration

Bei der stärksten in unseren Versuchen verwendeten Konzentration von NAA, nämlich 10^{-1} ‰ waren die Pflanzen extrem stark in ihrem Wachstum gehemmt. Die Länge des Hauptsprosses betrug bei den behandelten Pflanzen nur 64 mm (Mittelwert aus 5 Messungen) gegenüber einer Länge von 116 mm der Kontrollen. Am Stiel des Hauptsprosses, insbesondere am basalen Teil desselben, trat eine starke Adventivwurzelbildung in Erscheinung. Seitentriebe fehlten vollkommen und auch die morphologische Ausbildung der Blätter war, abgesehen von der allgemeinen starken Wachstumshemmung normal. In keinem Falle konnte Ringfasziation beobachtet werden. Es ist somit auffallend, daß selbst bei sehr hoher NAA-Konzentration, welche bereits nahe der Letal-Dosis gelegen war, zumal einige Pflanzen abstarben, keine Ringfasziation induziert wurde. Für die Induktion einer Ringfasziation scheint somit eine bestimmte Eigenschaft eines Wirkstoffes verantwortlich zu sein, welche der TIBA eigen ist, der NAA aber fehlt.

Bei der schwächeren NAA-Konzentration von 10^{-2} ‰ trat eine geringere Wachstumshemmung ein, morphologisch waren keine Veränderungen erkennbar. Dasselbe gilt auch für die NAA-Konzentration von 10^{-3} ‰ bei welcher jedoch eine geringe Förderung der Gesamtlänge festzustellen war.

Bezüglich der Beeinflussung der Gesamtlänge war somit auffällig, daß NAA, welche im *Avena*-Test bekanntlich eine starke Wuchsstoffwirksamkeit zeigt (z.B. Linser 1954) in unseren Versuchen mit Tomaten-Pflanzen nur bei 10^{-3} ‰ ganz schwach fördernd wirkte sonst aber das Wachstum hemmte. TIBA dagegen, welche im *Avena*-Test als reiner Hemmstoff in Erscheinung tritt, wirkte hier schwächer hemmend als NAA und bei einer Konzentration von 10^{-3} ‰ konnte sogar eine stärkere Förderung als die des Wuchsstoffes NAA festgestellt werden.

Die schwache Verzögerung der Blütenbildung bei NAA 10^{-2} ‰ (vergl. Tab. 1), durch welche die Blüten erst nach dem 9. Blatt des Haupttriebes erschienen, verdient deshalb Beachtung, zumal auch bei den nachfolgend zu beschreibenden Versuchen mit Mischungen von TIBA und NAA bei den stärkeren NAA-Konzentrationen eine geringe Verzögerung der Blütenbildung beobachtet werden konnte (vergl. Abb. 3).

c) Die Wirkung von TIBA in Gegenwart von NAA

Wie aus Tabelle 2 und Abbildung 3 ersichtlich ist, wirkten die Mischungen TIBA 10^{-1} ‰/NAA 10^{-1} ‰ sowie TIBA 10^{-3} ‰/NAA 10^{-1} ‰ letal. Auch bei der Mischung TIBA 10^{-2} ‰/NAA 10^{-1} ‰ waren von 5 behandelten Pflanzen nur zwei überlebend.

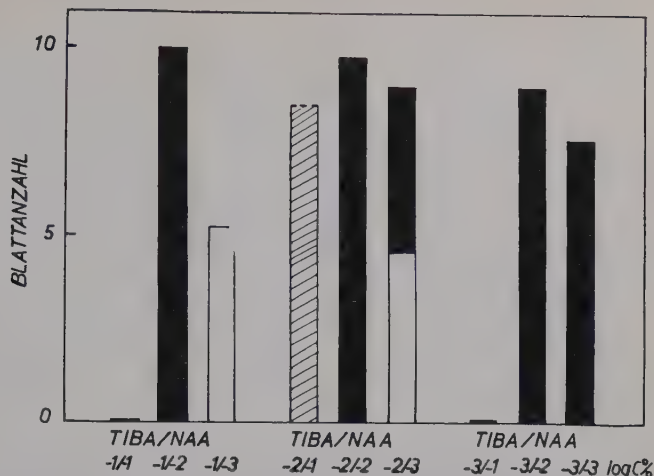


Abb. 3. *Lycopersicon esculentum* („Bonner Beste“). Wie Abb. 2, jedoch für Mischungen von TIBA und NAA.

Diese waren in ihrem Gesamtwuchs stark gehemmt (vergl. Abb. 4), zeigten starke Adventivwurzelbildung und hatten keine Seitentriebe ausgebildet. Die Blätter erfuhren ebenfalls eine starke Hemmung, waren jedoch morphologisch normal entwickelt. Ringfasziation konnte in keinem Falle beobachtet werden. Zum Zeitpunkt der Untersuchung waren noch keine Blütenstände entwickelt.

Eine auffallende Beeinflussung der morphoregulatorischen Wirksamkeit der TIBA war bei gleichzeitiger Zuführung von NAA in einer Konzentration von $10^{-2}\%$ zu beobachten. Während durch die Applikation von TIBA $10^{-1}\%$ allgemein Ringfasziation induziert wurde (vergl. Abb. 2) waren die Apices der mit einer Mischung von TIBA $10^{-1}\%$ /NAA $10^{-2}\%$ behandelten Pflanzen normal entwickelt und bildeten normale Infloreszenzen aus. Auch die vorzeitige Blütenentwicklung wie sie von TIBA $10^{-1}\%$ induziert wurde, wobei Blüten bereits nach dem 4,4 Blatt des Haupttriebes erschienen, wurde durch die gleichzeitige Anwesenheit von NAA $10^{-2}\%$ verhindert. An Stelle einer Verfrühung der Blütenentwicklung trat hier sogar eine leichte Verzögerung in der Blütenentwicklung ein, sodaß Blüten erst nach dem 9,8. Blatt des Haupttriebes erschienen. Die Mischung TIBA $10^{-1}\%$ /NAA $10^{-2}\%$ wirkte stärker hemmend auf die Gesamtlänge als die reinen Substanzen gleicher Konzentration, sodaß hier an eine synergistische Wirkung von TIBA und NAA gedacht werden kann. (Vergl. Abb. 4). Auch die formativen Veränderungen an den Blättern traten in Gegenwart von NAA weniger stark in Erscheinung.

Die Aufhebung der TIBA-bedingten Formbildungsänderungen durch NAA war bei der Mischung von TIBA $10^{-2}\%$ und NAA $10^{-2}\%$ am deutlichsten

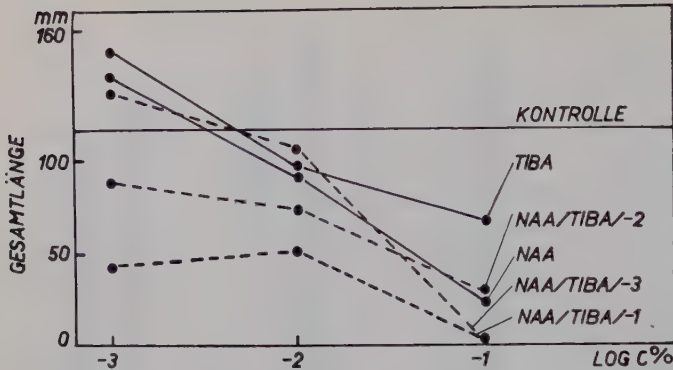


Abb. 4. Gesamtlänge des Haupttriebes von *Lycopersicon esculentum* („Bonner Beste“) nach Behandlung mit TIBA bzw. NAA und mit Mischungen von TIBA und NAA. Abszisse: Logarithmus der Konzentration in ‰, Ordinate: Gesamtlänge in mm.

sichtbar. Während bei TIBA $10^{-2}\%$ stets Ringfasziation an den Hauptsprossen zu beobachten war, hatten die mit der genannten Mischung behandelten Versuchspflanzen *vollkommen normale Infloreszenzen entwickelt*. Ringfasziation konnte bei den mit dieser Mischung behandelten Pflanzen in keinem Fall beobachtet werden. Die durch TIBA induzierte Verführung der Blütenbildung trat bei Anwesenheit von NAA $10^{-2}\%$ nicht mehr in Erscheinung. Eher war eine leichte Verzögerung zu beobachten. (Vergl. Abb. 3). Auch die starke Förderung der Seitentriebe durch TIBA $10^{-2}\%$ wobei Seitentriebslängen von 50—100 mm gemessen wurden (vergl. Tab. 1) wurde durch die gleichzeitige Anwesenheit von NAA $10^{-2}\%$ stark vermindert, sodaß nur noch Seitentriebe von maximal 10 mm Länge gefunden werden konnten (vergl. Tab. 2).

Hierbei fiel jedoch auf, daß sehr häufig Gamophyllie der ersten Blätter des Seitentriebes eintrat, welche zur Ausbildung von „Trichterblättern“ führte. Ob hier hinsichtlich der Ausbildung gamophyller Blätter ein Synergismus von Wuchs- und Hemmstoff herrscht, muß noch weiter untersucht werden. Jedenfalls zeigte sich auch bei der Untersuchung der Fiederanzahl pro Blatt eine ähnliche Wechselwirkung insofern, als bei gleichzeitiger Zuführung von Wuchs- und Hemmstoff eine wesentlich stärkere Reduktion der Fiederanzahl, die in Extremfall zu großen ungefederten gezähnten Blättern führte, eintrat, als bei Zuführung von TIBA allein.

Die starke teratogene Ausbildung der Blätter, wie sie bei TIBA $10^{-2}\%$ in Erscheinung trat, war durch NAA $10^{-2}\%$ stark gemindert. Gegenüber den reinen Stoffen gleicher Konzentration trat durch die Mischung TIBA $10^{-2}\%$ und NAA $10^{-2}\%$ eine etwas stärkere Hemmung des Längenwachstums ein (Abb. 4). Obwohl durch die Beimischung von $10^{-2}\%$ NAA die, durch $10^{-2}\%$ TIBA induzierte Ringfasziation und die Blütenförderung vollkommen gehoben wurde, war bei der teratogenen Ausbildung der Blätter aber auch

Tabelle 2. Die Wirkungen verschiedener Konzentrationen von TIBA auf einige morphologische Größen bei *Lycopersicon esculentum* („Bonner Beste“) in Gegenwart verschiedener Konzentrationen von NAA. N Normal; RF Ringfasziation; + leicht, ++ mäßig, +++ stark morphologisch verändert; Tr „Trichterblätter“.

Substanzgemisch	Log. C %	Gesamtlänge (mm)	Blatthabitus	Länge der Cotyledonatriebe (mm)	Blattanzahl ohne Cotyledonen bis zur Blüte	Ringfasziation	Bemerkungen
TIBA/NAA	—1/—1	—	—	—	—	—	{ alle Pflanzen letal geschädigt nur 1 Pflanze überlebend
TIBA/NAA	—1/—2	50	++	—	10	—	
TIBA/NAA	—1/—3	43	+++	40	5,2	RF	
TIBA/NAA	—2/—1	28	+	—	—	—	{ vegetativ, nur 2 Pflanzen überlebend
TIBA/NAA	—2/—2	72	+	10 (Tr)	9,8	—	
TIBA/NAA	—2/—3	88	++	105	4,6 (RF), (9)	RF	{ 3 Pflanzen mit RF 2 Pflanzen ohne RF alle Pflanzen letal geschädigt
TIBA/NAA	—3/—1	—	—	—	—	—	
TIBA/NAA	—3/—2	107	N	30	9,0	—	
TIBA/NAA	—3/—3	137	N	10—40	8,6	—	—

der Seitentriebe zwar eine bedeutende Abschwächung, jedoch noch immer ein Überwiegen der TIBA-Wirkung festzustellen. Dies ist erstaunlich, zumal NAA ein geringes Molekulargewicht (186,20) besitzt, wogegen TIBA ein solches von 499,55 aufweist. Bei der Mischung solcher prozentuell zwar gleich konzentrierter molar aber stark abweichender Lösungen im Verhältnis 1 : 1 wäre falls ein Antagonismus zwischen den beiden Stoffen herrscht und annähernd gleiche Affinität beider Moleküle zum Wirkungsort vorausgesetzt wird, ein stärkeres Hervortreten der Wirkung jener Substanz zu erwarten, welche das geringere Molekulargewicht aufweist, in unserem Falle also der NAA.

Die Mischung TIBA $10^{-2}\%$ /NAA $10^{-3}\%$, bei welcher somit der Anteil an NAA niedriger war, ergab dementsprechend eine stärkere morphoregulatorische Wirksamkeit. Während bei der stärkeren NAA-Konzentration die durch TIBA induzierte Ringfasziation vollkommen unterbunden wurde, zeigten hier von fünf Pflanzen nur drei Ringfasziation nach 4,6 Blättern des Haupttriebes, wogegen zwei Pflanzen normal ausgebildet waren und die Infloreszenz nach dem neunten Blatt entwickelt hatten (Abb. 3). Auch die morphologische Veränderung der Blätter sowie die Ausbildung von Seitentrieben entsprach dem starken Überwiegen der TIBA.

Die mit Mischungen von TIBA $10^{-3}\%$ /NAA $10^{-2}\%$ und TIBA $10^{-3}\%$ /NAA $10^{-3}\%$ behandelten Pflanzen waren normal ausgebildet. Bei der

Mischung TIBA 10^{-3} % / NAA 10^{-2} % war wieder eine schwache Verzögerung bei der Blütenbildung zu beobachten.

Zur orientierenden Untersuchung, ob die Beeinflussung der formativen Wirkung der TIBA durch NAA auch dann erfolgt, wenn beide Substanzen lokal getrennt, also nicht in unmittelbarer Mischung auf die Versuchspflanzen appliziert werden, wurde auf junge Tomatenpflänzchen mit Hilfe einer Mikropipette auf ein Keimblatt ein und derselben Pflanze 10 µg NAA, auf das andere 10 µg TIBA aufgebracht. Auch bei dieser Behandlungsart zeigte sich, daß NAA die morphoregulatorische Wirkung der TIBA weitgehendst abschwächt. Allerdings scheint der zeitliche Abstand der TIBA und NAA-Applikation bzw. die Reihenfolge der Behandlungen eine besondere Rolle zu spielen.

Diskussion der Ergebnisse

Die geschilderten vergleichenden Versuche an Tomatenpflanzen mit den Wirkstoffen 2,3,5-Trijodbenzoesäure (TIBA) und α -Naphthylessigsäure (NAA) haben u.a. gezeigt, daß 1. Ringfasziation nur durch TIBA, nicht aber durch NAA induziert werden kann und 2. daß die morphoregulatorische Wirkung der TIBA durch gleichzeitiges Zuführen von NAA kompensiert werden kann. Daraus erhebt sich die Frage, ob allgemein den Zellstreckungshemmstoffen, zu welchen die TIBA zählt, formative Eigenschaften zukommen, welche den Wuchsstoffen (z.B. NAA) fehlen. In dieser Richtung haben Versuche von Linser et al. (1957) und des Verfassers (1957, 1958) bei welchen eine größere Anzahl von Wuchs- und Hemmstoffen zur Untersuchung gelangten gezeigt, daß vor allem Hemmstoffe als Morphoregulatoren in Erscheinung treten, wobei jedoch wie Kiermayer und Youssef (1957) hinwiesen, nicht alle Stoffe welche stark zellstreckungshemmend wirksam sind, auch formative Effekte auslösen können. Gegen die Annahme, daß vor allem eine bestimmte Gruppe von Hemmstoffen formativ wirksam ist, scheinen Versuche von Laibach und Mai (1936) und Ball (1944) zu sprechen, bei welchen auch Wuchsstoffe (Indol-3-essigsäure) starke morphologische Veränderungen hervorriefen. Demgegenüber weist jedoch Wenck (1952) darauf hin, daß bei den Arbeiten der genannten Autoren sehr hohe Wuchsstoffdosen zur Anwendung gelangten, wobei sehr wahrscheinlich schon die Hemmkomponente des Wuchsstoffes wirksam wurde. Eine starke Unterstützung der Ansicht, daß die Hemmkomponente (Linser, 1955) gewisser Stoffe für die formative Wirksamkeit verantwortlich ist, erfolgt nun durch die geschilderten Versuche mit Mischungen von TIBA und NAA. Es scheint, daß zwischen gewissen Wuchs- und Hemmstoffen auch hinsichtlich ihrer morphoregulatorischen Wirksamkeit eine ähnliche gegenseitige Beeinflussung möglich ist, wie dies für Zellstreckungsvorgänge bekannt ist. (Linser 1951, 1954; Linser u. Kaendl, 1951.)

Dafür sprechen auch die schon erwähnten Versuche von Wenck (1952) an *Codiaeum variegatum*. Ob es sich um einen echten Antagonismus handelt, kann aus den wenigen bisherigen Versuchen nicht mit Sicherheit gefolgert werden. Vielmehr müssen noch folgende mögliche Ursachen für eine gegenseitige Wirksamkeitsbeeinflussung von Wuchs- und Hemmstoffen mitberücksichtigt werden:

- 1.) Die Aufhebung der hemmstoffbedingten formativen Wirkung könnte durch eine bereits *in vitro* erfolgende Inaktivierung des Hemmstoffes durch den Wuchsstoff erfolgen. Diese vorerst naheliegende Vermutung dürfte jedoch auszuschließen sein, da (wie orientierende Versuche gezeigt haben) auch dann eine Aufhebung der hemmstoffwirkung erfolgt, wenn Wuchsstoff und Hemmstoff lokal voneinander getrennt auf die Versuchspflanzen appliziert werden.
- 2.) Eine Blockade des Transportes an endogenem Wuchsstoff durch TIBA wie sie unter anderem von Kuse (1953) und Hay (1956) angenommen wird und die damit verbundene mangelhafte Wuchsstoffversorgung der Vegetationsspitze könnte durch zusätzliches Zuführen von Wuchsstoff kompensiert werden.
- 3.) Die bedeutende Herabsetzung der Wachstumsgeschwindigkeit durch NAA könnte dazu führen, daß die in die Pflanze gleichzeitig eingedrungene Menge an TIBA bis zum Erreichen des sensiblen Stadiums des Vegetationspunktes schon soweit abgebaut ist, daß die verbliebene Menge an TIBA nicht mehr ausreicht um noch morphoregulatorisch wirksam zu sein.

Zusammenfassung

Bei vergleichenden Untersuchungen mit TIBA und NAA an *Lycopersicon esculentum* konnte folgendes festgestellt werden:

1. TIBA induzierte an den Tomatenpflanzen verschiedene teratogene Effekte, während NAA in höherer Konzentration zwar stark wachstumshemmend, jedoch ohne morphoregulatorische Wirksamkeit gefunden wurde.
2. Die teratogene Wirkung der TIBA konnte durch gleichzeitiges Zuführen von NAA entsprechend dem Mischungsverhältnis abgeschwächt, bzw. vollkommen aufgehoben werden.
3. Bei gleichzeitigem Zuführen von NAA wurden folgende TIBA-induzierbaren Bildungsabweichungen abgeschwächt bzw. vollkommen verhindert:
 - a) Ringfasziation
 - b) Förderung der Blütenbildung
 - c) Austreiben der Seitentriebe
 - d) Morphologische Veränderung der Blätter.

4. Eine möglicherweise synergistische Wirkung der Mischung TIBA/NAA wurde hinsichtlich der Wachstumshemmung, der gamophyllen Blattausbildung, sowie der Blattfiederreduktion beobachtet.
5. Es wird vermutet, daß die Hemmkomponente eines Wirkstoffes bzw. eine mit dieser Komponente gekoppelt auftretende Wirkung für die morphoregulatorische Aktivität verantwortlich ist. Die Wuchsstoffkomponente scheint diese Wirkung in entgegengesetztem Sinne zu beeinflussen.

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A Role of the Laccase of Wood-rotting Fungi

By

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Introduction

The laccase of white wood-rotting fungi is associated with the decomposition of lignin (Freudenberg 1959) but evidence is lacking concerning its role in this process. Some lignins which have been extracted and treated by chemical means are unreactive as substrates, while others used by Dion (1952) showed some oxygen uptake in the presence of the enzyme. Higuchi (1956) has demonstrated activity with decayed beech wood meal due to the presence of phenolic substances, and Gadd (1957) stated that the function of the enzyme is oxidation and polymerization of toxic compounds liberated during the decomposition of lignin.

The present work concerns a further investigation of the role of the laccase using both lignin residues and sound wood as substrates. Tests have also been applied to determine the fate of the oxidation products of simple phenols.

Materials and Methods

The enzyme. *Polystictus sanguineus* (L.) was grown on 100 ml. of medium in conical flasks or Roux bottles. After 3—4 weeks the mycelium was filtered off and the medium dialysed against distilled water.

<i>Medium</i>	Glucose	30	gm.	<i>Medium</i>	MgSO ₄	0.5	gm.
	Marmite	2	gm.		FeSO ₄	0.01	gm.
	KH ₂ PO ₄	1	gm.		ZnSO ₄	0.01	gm.
	NaCl	0.5	gm.		CaCO ₃	0.1	gm.

Distilled water to 1 litre.

Lignin and Wood Samples. Lignin was obtained from sections of Scots pine sapwood which were inoculated with *Merulius lacrymans* and allowed to rot for twelve months. The average loss in weight was 46.5 %. The sections were ground and sieved finely. Sections of the original sound wood, averaging 6 gms. in weight were kept as controls. These were shaved finely with a file and sieved. Lignin from *Pinus contorta* sapwood after the action of *Poria monticola* was also used as substrate. Samples of the sound wood were shaved and sieved.

Manometric procedure. Oxygen uptake was measured using the following conditions: 0.3 ml. of citric acid-phosphate buffer (McIlvaine) and 1.5 ml. of medium were added to the centre and 0.2 ml. 10 % KOH to the centre well. The substrate in a volume of 0.5 ml. was added to the side arm, the total volume being 2.5 ml. The flasks were equilibrated with air and the experiments carried out at 25°C.

Substrates — Catechol 5 mg. Lignin (solid) 20 mg.
 Guaiacol 5.6 mg. Lignin extract 4 %

The extract was prepared by heating the lignin in water at 60°C for one hour, centrifuging and filtering. The final concentration in the manometric system is approximately equivalent to 20 mg. of the solid.

Chromatography. Solvents: (1) Butanol 40: Glacial Acetic Acid 10: Water 50.

(2) Pentanol 20: Ethanol 12: Water 10.

Sprays: (1) Ammoniacal silver nitrate.

(2) Benzidine reagent (Lindstedt 1950).

Results

Lignin. The action of the laccase of *Polystictus sanguineus* on some phenolic substrates has already been studied (Law 1955). In the present work, guaiacol as well as catechol has been used and the course of oxidation of these substrates is illustrated in Figure 1. Lignin isolated by chemical means was insoluble in water and not oxidized. However the material remaining

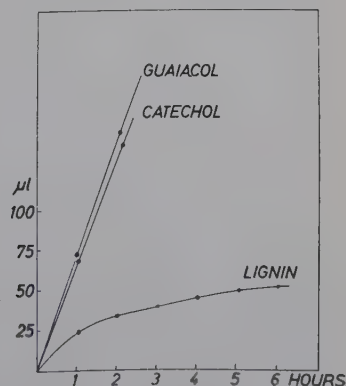


Figure 1. Action of laccase on guaiacol (pH 4.0), catechol (pH 4.5) and lignin extract (pH 5.5). Controls with boiled enzyme, nil. On the ordinate μl O_2 uptake.

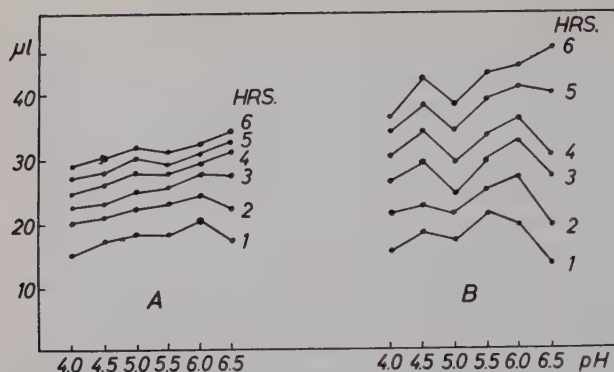


Figure 2. Action of laccase on lignin over the pH range 4–6.5 at hourly intervals for 6 hours. On the ordinate $\mu\text{l O}_2$ uptake. (A) Lignin extract. (B) Lignin suspension.

after the action of *Merulius lacrymans* or *Poria monticola* on wood, is partly soluble in water, the soluble fraction giving common phenolic tests. This solution was used as substrate for the enzyme. The uptake of oxygen is slow and goes on for a long period (Figure 1), the resultant solution becoming darker in colour. The action proceeds over a wide range of pH (Figure 2) and a definite optimum point is unobtainable. Numerous experiments on different lignin samples gave similar results indicating that the material contained a number of oxidisable substances or that more than one enzyme participated in the reaction.

Enzyme resulting from growth on medium containing 1 gm. of lignin per 100 ml showed the same action. Dialysis of the media did not effect the activity of the enzyme. In most experiments, as illustrated in Figure 2 the course of the action of the enzyme on the solid followed a slightly different pattern from that on the extract, possibly due to the low solubility of certain of the phenols. The main structure of the lignin appeared to be unaffected by the enzyme, since removal of the phenolic substance by refluxing for several hours left a residue which was not oxidizable.

Chromatograms were run to investigate the components of the substrate system. About 0.1 ml. of 10 % lignin extract was placed on No. 1 Whatman filter paper and the solvent allowed to move upwards for 16 hours. Butanol-acetic acid and pentanol-alcohol were used as solvents and similar results were obtained in each of these, though streaking of the lignin was less in the pentanol-alcohol and R_F values in this solvent are quoted. The chromatograms were sprayed with the benzidine reagent, followed by 2 % sodium bicarbonate solution. In earlier experiments duplicates were sprayed with ammoniacal silver nitrate but the benzidine reagent was more satisfactory and therefore adopted. Most of the spots developed rapidly and began to fade after 10 minutes. Some were large and spreading and the R_f values given

below were measured to the centre of the spot. The colour given with the reagent is indicated. Over the whole there was always a certain amount of brown streaking but the spots were clearly superimposed on this.

R_f — inches.

Scots pine lignin extract			<i>Pinus contorta</i> lignin extract		
1.	0.70	pink	0.82	pink	
2.	0.63	brown	0.70	brown	
3.	0.56	pink	0.60	pink	
4.	0.45	pink	0.45	pink	
5.	0.30	brown	0.36	brown	
6.	0.24	brown	0.20	pink	
7.	0.17	pink	0.13	brown	

Tests were carried out as follows to demonstrate the effect of the enzyme on the substances represented by the spots.

20 % lignin extract	3	ml.
enzyme	3	ml.
pH 4, 5, 6 buffer	0.5	ml.
merthiolate	0.001	M (final concentration)
Incubated at 30°C.			

Samples were removed at 24, 48 and 60 hours and compared with controls containing boiled enzyme. After 48 hours spot No. 1 had disappeared at pH 5 and pH 6. After 60 hours Nos, 2, 3, 5, 6, and 7 had disappeared at pH 6 and were faint at pH 5. At pH 4 the difference was less marked compared to the controls which showed the original pattern throughout. Spot No. 4 remained, though it diminished at all pH values.

Wood. Extracts of Scots pine and of *Pinus contorta* were made by boiling 10 gm. of the wood in 120 ml. water under a reflux condenser for several hours. Phenolic substances are contained in the extracts and differ from those of heart-wood of various species (Lindstedt 1950). The R_F values for the two species measured to the centre of the spots are tabulated and the resultant colour with the benzidine reagent indicated.

R_f — inches.

Scots pine extract			<i>Pinus contorta</i> extract.		
1.	0.73	brown	0.96	pink	
2.	0.50	brown	0.75	brown	
3.	0.14	pink			
		residue pink.			

The action of the enzyme on these phenolic substances is too slow to follow manometrically. The procedure described immediately above for lignin was repeated with these extracts. After a few hours a slight change in colour and an increase in turbidity were noticeable. In the case of Scots pine spots Nos.

1 and 2 disappeared in the presence of the enzyme at pH 4, 5, and 6 in 48 hours, and spot No. 3 disappeared at pH 6 only. The *Pinus contorta* extract was affected more slowly. Spot No. 1 disappeared at pH 6 only in about 100 hours. Spot No. 2 had disappeared in the same time at all pH values. The boiled controls remained unaltered throughout.

Experiments on the wood and lignin have indicated that the enzyme oxidized these phenolic substances without affecting the main structure of the lignin. Samples soaked in enzyme solution at various pH values still reacted with phloroglucinol after several weeks. When *P. sanguineus* was grown on medium containing wood or lignin, there was no detectable change in the phloroglucinol reaction after three months.

Fate of the phenolic substances. The experiments described, in which lignin extracts were incubated with enzyme, indicate that the phenols are oxidized to coloured products, probably quinones. A similar action occurs with phenols such as catechol and guaiacol which are oxidized to brown and red solutions respectively. These solutions contain nondialysable polymerized products and show a marked residue in the initial spot on a chromatogram. Addition of a reducing agent such as ascorbic acid shows no effect on these end products. However if ascorbic acid is added shortly after oxidation has commenced, the colour is immediately discharged, suggesting that simple quinones formed early in the oxidation are reduced.

That the mycelium might be capable of reducing these quinones was first indicated by changes observed during growth of *P. sanguineus* on agar plates containing certain phenols. Petrie dishes containing 2.5 % agar in the medium were prepared as usual. Catechol and guaiacol solutions were made in sterile water and added after autoclaving. The final concentration of the phenols ranged from 0.05 mg./ml.—0.1 mg./ml. Controls without phenol were also prepared and all the plates were inoculated and incubated at 30°C. The growth of the fungus was normal and as it proceeded, the quinone colour appeared in the agar. Ten or twelve days after inoculation this colour lightened and in places disappeared leaving only patches. A similar effect occurred when the agar contained 10 % lignin extract.

Agar plates containing preformed quinones (phenols oxidized by the enzyme for 16—24 hours) of approximately the same concentration gradually decolourized as the mycelium grew. The change was slow and after four weeks some areas of colour still remained.

In order to investigate these changes, homogenates of actively growing mycelium 14—16 days old, were prepared. It was washed free of medium, but at this stage still contains a high concentration of enzyme. Tests were set up in 10 ml. centrifuge tubes as follows and incubated at 30°C.

Mycelium (average dry weight 20 mg/ml) pH 4.5—5	5	ml
Catechol or guaiacol 0.1 %	0.2	ml.
or		
Quinone preformed with enzyme	3.5	ml.
Sodium azide (when added) — final concentration	0.001	M
Volume made to 10 ml.		

Controls with boiled mycelium.

Tests containing sodium azide were included in each series.

(Owing to its instability several additions of sodium azide are necessary during the long periods).

The phenols were oxidized to coloured products by the enzyme unless sodium azide had been added but after about an hour decolourization proceeded rapidly, though shaking restored the colour temporarily. Tubes were removed at 24, 48 and 72, hours and centrifuged. Chromatograms of the supernatant of controls and tests were run, using 100 μ l. of each. The catechol and guaiacol had disappeared from the supernatant of the tests in 24 hours but were present in the controls and in tubes containing sodium azide. Since enzymic oxidation of small amounts of phenolic substances occurs easily, sodium azide was added to some of the samples before applying the spots. In the case of guaiacol which is very volatile, 200 μ l. were added to the paper and the chromatograms run for only four hours. No indication of the presence of catechol or guaiacol was given under these conditions in the tests. When sodium azide had been added to inhibit the enzyme, the phenol showed clearly on the chromatogram.

After removal of the supernatant, the mycelium was extracted in a small amount of alcohol and ether and the extracts made to the original volume with ether. Chromatograms were run using the same amounts as for the supernatants. The extracts were then concentrated to a small volume which was all put on the paper. In the early periods the phenols were identified only in the concentrated extracts. At 72 hours the spot was faint or often not visible even when concentrated. Incubation with enzyme for several hours before addition of the mycelium facilitated the disappearance of the catechol in both supernatant and mycelium. Tests carried out under anaerobic conditions were similar though decolourization proceeded more effectively.

In a second series preformed quinones were used in place of the phenols. The quinone was slowly decolourized and both supernatant and mycelium were light in colour in 24—48 hours. In the controls the quinone was adsorbed by the mycelium but not decolourized. No evidence of the presence of the phenol was obtained in either supernatant or mycelium. In tests where sodium azide was added, the decolourization proceeded at the same rate which suggests that the laccase is not involved in the mechanism responsible for changes in the quinone.

Discussion

In considering the association of laccase with lignin decomposition, the only role for which there is evidence is that of oxidation of associated phenolic substances. The use of a natural substrate has provided confirmation of this. Samples of Scots pine rotted by *Merulius lacrymans* and of *Pinus contorta* rotted by *Poria monticola* both yield a mixture of phenolic substances differing from those in the original wood. The changes that occur due to the action of these fungi are not within the scope of the present investigation and no attempt has been made to identify the components of this substrate system. The oxidation of most of these substances by the laccase of *Polystictus sanguineus* has been demonstrated. It is probable that the mixture includes vanillic and syringic acids, vanillin, and syringic and coniferyl aldehydes as identified by Higuchi (1956) in a similar type of wood meal. Henderson (1955) showed that vanillic and syringic acids were liberated also by white rots when the latter were grown on certain sawdusts. The experiments reported here indicate that the medium containing laccase also produces changes in the phenols of sound wood and a certain proportion disappears. However, the main bulk of the lignin, judging by the general structure and the phloroglucinol reaction, is little affected by this enzyme throughout long periods.

Freudenberg *et al.* (1958) and Higuchi (1957) have demonstrated that certain laccases oxidize and polymerize phenolic substances to materials resembling lignin. As already stated, Gadd (1957), attributes to the laccase of white rots, the role of oxidation and polymerization as a mechanism for the removal of toxic monomeric aromatic substances. In the presence of the enzyme alone, coloured, undialysable, polymerized products can result, as demonstrated with catechol, guaiacol and lignin extracts. However, the sequence of events does not always lead to polymerization. The intermediate coloured products of the reaction, probably simple quinones, disappear in the presence of the mycelium. The decolorization is reversible and suggests a reduction but the disappearance of the quinone colour was not accompanied by the appearance of a phenol. Where sodium azide was added, the phenol remained in the medium. It may be assumed from these observations that the phenols are oxidized to quinones as a first step in their utilization by the mycelium and may play a part in a variety of mechanisms and syntheses (Mason 1955). Excess products which remain in the medium are polymerized, accounting for permanent coloured patches in experimental plates and in sections of wood.

The factors involved in the degradation of lignin still remain obscure and

though white rots, by virtue of their laccase, may utilize phenolic substances derived from lignin, the exact role of this enzyme cannot yet be defined.

Summary

The laccase of *Polystictus sanguineus* oxidizes a number of phenols in the lignin residue remaining after the action of certain fungi on wood. The main lignin structure appeared to be unaltered after long periods of contact with laccase. Phenolic substances in sound wood are also oxidized.

Observations on the fate of the oxidation products of guaiacol and catechol indicate that one of the roles of this laccase may be oxidation of phenolic substances for utilization by the fungi.

I am indebted to the Forest Products Research Laboratory, Princes Risborough, for supplying the cultures and samples of wood and lignin.

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Chemical activity of the glucose adduct of 3-amino-1,2,4 triazole

By

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The results of chromatographic and tracer techniques indicate that the herbicide, 3-amino-1,2,4-triazole (3-AT) is metabolized by plants (5, 6). The product common to all plants treated with radioactive 3-AT has been identified as the glucose adduct (6). Chromatographic analyses of extracts from plants treated with this adduct (8) indicate that it too may be metabolized by the plant.

The ability of 3-AT to form a stable complex with glucose suggests one possible mechanism for its growth-inhibiting properties. The formation of the glucose-3-AT adduct could conceivably make a considerable quantity of glucose unavailable for normal metabolism. It is equally possible that the adduct or a derivative may act as an inhibitor in various enzymatic reactions in which glucose or a product of glucose metabolism is a substrate.

This paper deals primarily with the properties of the glucose adduct of 3-AT and its participation in the hexokinase reaction.

Materials and Methods

The glucose derivative of 3-AT has been prepared by allowing a mixture of 3-AT and glucose in water to stand at room temperature for several days. The product obtained was presumably the amine glucoside with a molecular weight of 247 (1). For our experiments, the glucose-3-AT complex was prepared according to Sund (8) by adding 0.25 moles of 3-AT dissolved in a minimum amount of 95 % ethanol to

Table 1. *A comparison of some of the physical and chemical properties of glucose, 3-AT, and the glucose adduct of 3-AT.*

M.P.	Glucose 146°C.	3-AT 156—7°C.	Glucose-3-AT adduct 215°C. (dec.)
Ninhydrin test	(—)	(+) dark violet	(—)
Fehling's reagent	(+)	(—)	(—)
Tollen's reagent	(+)	(—)	(—)
Schiff's reagent	(+)	(—)	(—)
Ferric chloride	(—)	dark red chelate	(—)
Rf		0.50	0.14

0.25 moles of glucose dissolved in a minimum amount of water containing 1 g. of sodium acetate. The solution was heated in an oven at 50—60°C. for about 30 hours. The product which was formed after the solution was cooled to room temperature was removed by filtration and washed several times with hot methanol. The method described by Sund (8) was used for the purification of 3-AT. A saturated solution of 3-AT in hot ethanol containing activated charcoal was filtered and the 3-AT crystallized on cooling. The final product was recrystallized from 2-propanol. Yeast hexokinase, crude, type II (Sigma Chemical Co.) was used where indicated and hexokinase activity was measured manometrically by the method of Colowick and Kalckar (2). With this method, one μM of CO_2 is released for each μM of phosphorus transferred to glucose.

Results

In order to test for the purity of the glucose adduct of 3-AT and to obtain information necessary for determining its structure, the compound was subjected to a number of chemical tests, along with glucose and 3-AT (Table 1). The melting point and chromatographic data are obtained from Sund (8) and clearly indicate that the adduct is indeed a distinct compound. This conclusion is also supported by the infrared absorption spectra (8). Further the absence of a positive ninhydrin reaction indicates that the free amino group, present in 3-AT, is blocked in the adduct. Evidence is presented to show that the free aldehyde group of glucose is also blocked in the adduct. The failure of the adduct to form a dark red chelate with ferric ions indicates that the groups which enter into the ferric ion-3-AT reaction (3) are also blocked. It is suggested that the adduct is formed by bonding of the amino group of 3-AT to the number 1 carbon of glucose. In order to form the adduct in this manner, the aldehyde oxygen of glucose would react with the two hydrogens of the 3-amino group of 3-AT, splitting off a molecule of water. This compound, the amine glucoside, would have a molecular weight of about 247, and has been suggested as the one most likely to be formed (1).

To explore the possibility that the formation of the glucose-adduct of 3-AT

Table 2. *Phosphorylation of glucose and the glucose-3-AT complex by yeast hexokinase.*

The main compartment of each Warburg vessel contained 0.02 *M* NaHCO₃, 0.01 *M* MgCl₂, 0.5 mg. hexokinase, 0.02 *M* substrate, and when added, 0.02 *M* 3-AT, total vol. 1.2 ml., pH 7.5. Side arm contained 0.04 *M* ATP (sodium salt, pH 7.5), 0.2 ml.; 0.04 *M* NaHCO₃, 0.2 ml. Reaction time, 20 mins. Gas phase, 95 % N₂—5 % CO₂; Temp. 30°C. The contents of the side arm were added to the main compartment at zero time. Data are corrected for CO₂ evolution in the absence of substrate.

Substrate	μl CO ₂ evolved
glucose	119.3
glucose + 3-AT	112.3
glucose-3-AT complex	51.1
3-AT	0

prevents further metabolism of glucose, both glucose and the glucose adduct of 3-AT were utilized as substrates in the hexokinase reaction. The results presented in Table 2 indicate that the glucose adduct acts as a substrate for hexokinase, but the rate of the reaction is considerably less when compared to an equimolar concentration of glucose (0.02 *M*). The glucose adduct is quite stable and did not break down under the conditions of these experiments. When 3-AT was added to the reaction mixture, there was no significant reduction in the amount of glucose phosphorylated, and the adduct did not form under the conditions of this experiment. The addition of 3-AT to a reaction mixture lacking substrate showed no increase in CO₂ evolution as compared to controls.

In order to further investigate the participation of the glucose adduct of 3-AT in the hexokinase reaction, the effect of substrate concentration on hexokinase activity was measured. The results (Figure 1) indicate that,

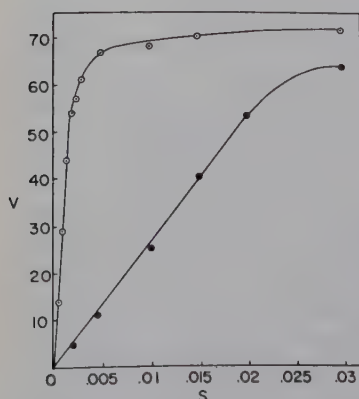


Figure 1. *The effect of substrate concentration on hexokinase activity. S=substrate concentration (molar); V=velocity of reaction (μl CO₂ evolved); reaction time, 10 mins. Substrates: glucose, upper curve; glucose adduct of 3-AT, lower curve. Reaction mixture as in Table 2.*

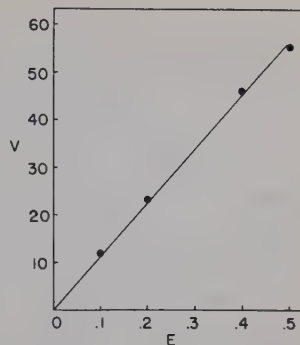


Figure 2. The effect of enzyme concentration on hexokinase activity. Substrate, glucose adduct of 3-AT. E=mg. hexokinase, V=velocity of reaction ($\mu\text{l CO}_2$ evolved). Reaction time, 10 mins. Reaction mixture as in Table 2.

although the adduct acts as a substrate for hexokinase, the affinity of this compound for the enzyme was considerably less than that of glucose. Within the concentration range indicated (Figure 2), the rate of the hexokinase reaction with the glucose adduct of 3-AT as substrate was directly proportional to the concentration of hexokinase.

Discussion

Although 3-AT has been shown to form an adduct with glucose both *in vitro* and *in vivo*, the possible connection between this reaction and the herbicidal properties of 3-AT has not been discussed. There is a real possibility that once glucose has become attached to 3-AT, it is effectively removed as a substrate for many enzymatic activities in the cell. The unavailability of this primary energy source could in turn affect many synthetic processes in the plant. In view of this possibility we have explored the effects of 3-AT and the glucose adduct of 3-AT on the hexokinase reaction. This reaction is not affected by 3-AT, although 3-AT is capable of chelating metals (1, 7) and forms a solid complex with magnesium $[\text{MgCl}_2 (\text{3-AT})_4 \cdot 8\text{H}_2\text{O}]$ (1). However, it does not necessarily follow that 3-AT should chelate the magnesium of hexokinase. If the stability constant is greater for the hexokinase-Mg chelate than the 3-AT-Mg chelate, the 3-AT would not chelate the Mg of this enzyme. However, 3-AT has been demonstrated to inhibit phosphorylase activity, an inhibition which can be reversed by the addition of manganese to the system (3).

The glucose adduct of 3-AT acts as a hexokinase substrate, although its affinity for the enzyme is considerably lower than glucose. If large enough amounts of glucose are complexed by 3-AT in plants, it is possible that the amount available for the first step in glycolysis may be significantly reduced.

Although the adduct itself may react with hexokinase, the low affinity of this substrate for the enzyme makes it unlikely that any significant quantities will be metabolized. It is not surprising that the glucose adduct of 3-AT can be utilized as a substrate in the hexokinase reaction since it has been shown that hexokinase does not have an absolute requirement for any of the groups as they exist in the glucose molecule. Although many compounds differing in steric configuration from glucose may serve as hexokinase substrates, the affinity of such compounds for the enzyme is greatly reduced (4). It is not known whether the phosphorylated glucose adduct of 3-AT undergoes further metabolism in the plant, although it has been reported that the glucose-adduct itself, when applied to plants, cannot be detected in plant extracts (8). It is possible that the adduct, once it has been phosphorylated, may be involved in other reactions. It is equally possible that the phosphorylated adduct or one of its derivatives may act as an inhibitor to other enzymatic reactions. Definite conclusions regarding the physiological significance of the formation and metabolism of this compound await further investigation.

Summary

1. The herbicide, 3-amino-1,2,4 triazole forms an adduct with glucose both *in vitro* and *in vivo*. Evidence presented indicates that this adduct is the amine glucoside.
2. Although 3-AT does not affect hexokinase activity, the glucose adduct acts as a substrate for this enzyme. The affinity of the adduct for hexokinase is considerably lower than glucose.
3. The possible physiological significance of the formation of the 3-AT-glucose adduct is discussed.

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Essential Metal Requirements of *Oscillatoria* phosphorylase

By

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The necessity for a divalent metal ion for *Oscillatoria* phosphorylase action has been reported previously (Fredrick 1954). The possibility that this cation might be *manganese* was postulated by Fredrick (1958, 1959) and by Fredrick and Gentile (1959). The *chelate* nature of this phosphorylase-metal bond was explored in previous reports from this laboratory (Fredrick 1957, 1958, 1959). However, because exhaustive dialysis of this algal phosphorylase did not significantly affect its activity in synthesizing polyglucoside from substrates of glucose-1-phosphate (Fredrick 1954), it was not possible until now, to obtain a suitable preparation of this enzyme with which to study its specific metal ion requirements.

In view of this, it was decided to adapt the methods used by Schubert (1948) and by Schubert and Richter (1948) for the determination of chelate stability constants by ion exchange resins, to the problem of obtaining a metal-free (and hence, an activity-free) preparation of *Oscillatoria princeps* phosphorylase. Many different types of ion exchange resins were evaluated for this task, but the best results were obtained using a resin comprised of styrene-divinylbenzene matrix within which were attached iminodiacetate groups (Dowex A-1, Dow Chemical Co.). It was found, by comparative studies, that this resin did not adsorb significant quantities of the phosphorylase as did the other types of cation exchange resins tried. Also, of decided advantage was the fact that this resin effectively removed the chelate-bonded phosphorylase metal upon short periods of contact and in the cold.

Experimental

Algal phosphorylase was prepared from healthy cultures of *Oscillatoria princeps* after the modification of the method of Fredrick and Mulligan (1955) by Fredrick and Gentile (1959). The enzyme solutions contained approximately 10 mg. of protein per ml. of 0.001 *M* Tris buffer at a pH of 7.2.

The ion exchange resin (Dowex A-1) was put into the sodium form as per directions (Dow 1959). It was then washed with cold distilled water (10°C) until completely free of "color throw", and then was stored wet in a refrigerator at 6°C until just prior to use. All subsequent procedures were performed at 6°C.

15 ml. of the cold resin slurry were added to 50 ml. of the enzyme solution. This mixture was shaken for 1 hour and then the resin was separated by filtration on Whatman no. 40 paper. The opalescent filtrate was used in the tests described below. It will be referred to as *resin-treated* enzyme.

Calculations based on the findings of Fredrick and Gentile (1959) that a 50 per cent inhibition of *Oscillatoria* phosphorylase was obtained with a concentration of chelating agent (in that case, 3-aminotriazole) theoretically capable of removing 5×10^{-4} *M* concentrations of divalent metal ions from exactly 1.0 ml. of the enzyme solution, indicated the desirability of starting these studies with low concentrations of these ions. Therefore, manganese ion, as the chloride, was preincubated at 26°C with 1.0 ml. of resin-treated enzyme solution in Tris buffer at pH 7.2 for 1 hour. (At this concentration of Tris buffer (Tris (hydroxymethyl) aminomethane), there is no interaction between the buffer and manganese ion (Fredrick and Gentile 1959, Fredrick 1959 b)). This mixture was then added to a reaction mixture made up of 0.05 *M* dipotassium glucose-1-phosphate (Schwarz Labs., Mt. Vernon, N.Y.), 0.1 per cent amylose (Merck) and 0.001 *M* Tris buffer in a total volume of 10.0 ml. and at a pH of 7.2, and was incubated at 30°C for 36 minutes (Fredrick and Gentile 1959).

The concentrations of manganese ion used for the enzyme preincubation ranged from 10^{-6} *M* through ten-fold increments to 2 *M*. Parallel experiments were set up using magnesium chloride, calcium chloride and ferrous sulfate in the same concentrations.

All data are based on the amount of inorganic phosphate released from the substrate at the end of 36 minutes. The activity of the resin-treated enzyme was compared with that of untreated enzyme from the same preparation. The activity of the untreated enzyme was taken as representative of 100 per cent activity. As a further check, the polysaccharide formed in the reaction mixtures was precipitated, washed, hydrolyzed with 2 *N* hydrochloric acid, and the reducing power determined as glucose (Fredrick 1951).

Results

In all cases, the reducing power of the polysaccharides formed compared substantially with the inorganic phosphorus released from the substrate by phosphorylase action. Magnesium, calcium and ferrous ions were without effect.

As can be seen in Figure 1, even with no added metallic ion there still remained some residual phosphorylase activity in the *resin-treated* enzyme preparation.

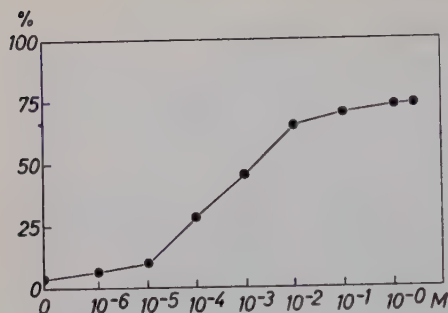


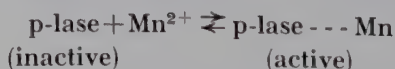
Figure 1. Effect of increasing concentrations of manganese on phosphorylase of *Oscillatoria*. Ordinate shows per-cent of total activity as compared to non-resin treated control. Abscissa shows concentration (molar) of manganese ion used.

With the lower concentrations of manganese, there was a slight increase in the activity of *resin-treated* phosphorylase. As the manganese ion concentration increased above 10^{-5} M however, there was a rapid increase in phosphorylase activity which persisted with increasing concentrations of the ion until a leveling-out was obtained at a concentration of manganese of 10^{-2} M. Further increases in concentration of this ion had slight, if any, effect.

Some denaturation or other loss (adsorption on the resin of the phosphorylase had occurred as can be seen (cf. Figure 1) from the fact that the maximum activity of the *resin-treated* enzyme preparation was about 75 per cent of that of the untreated enzyme.

Discussion

The evidence presented in Figure 1, of increasing activity of algal phosphorylase with increasing concentrations of manganese ion, seems to substantiate the existence of the following reaction postulated by Fredrick (1957, 1958, 1959):



whereby, in order for the phosphorylase protein to manifest polysaccharide-synthesizing activity, it was essential that it formed a *chelate* with manganese. In this respect, this algal phosphorylase behaves much like the leucine aminopeptidase studied by Smith and Bergmann (1946) which also required manganese ion for enzyme activity. In fact, further similarities are apparent if one compares the activity curves of these two enzymes (cf. Figure 1 and Smith 1946). It is tempting to assume that the controlling agent of these two

"different" enzymes is the metallic ion rather than the specificity of the particular proteins concerned.

Manganese has also been shown to be necessary for the conversion of rabbit muscle phosphorylase *b* (inactive without added adenosine monophosphate) to the active phosphorylase *a* form (Krebs and Fischer 1956). It is interesting that the maximum conversion of the *b* to the *a* form occurred at a manganese concentration of 10^{-3} *M* (Krebs and Fischer 1956). As can be seen from Figure 1, this concentration of manganese is also optimum for *Oscillatoria* phosphorylase.

It is possible to extrapolate certain considerations as to the strength of the phosphorylase protein-manganese bond from the figure. For example, where the activity is exactly 50 per cent of possible activity (in this case, the maximum possible activity is about 75 per cent, and midway would be about 37.5 per cent on the curve shown in Figure 1), the following condition probably exists:

$$(\text{p-lase protein}) = (\text{p-lase enzyme})$$

and hence, the metal positions (Smith 1946) on the protein are about half occupied. It is then possible to determine the log of the formation constant of the phosphorylase-manganese chelate from this point:

$$K = (\text{p-lase enzyme}) / (\text{p-lase protein}) (\text{Mn}^{2+})$$

Since (p-lase Protein) is equal to (p-lase Enzyme) at the point specified, then the value of log *K* becomes log $1/(\text{Mn}^{2+})$. This, read directly from the curve in Figure 1 is about 3.6.

Conclusions

1. A metal-free, inactive preparation of algal phosphorylase was obtained by treating active *Oscillatoria princeps* enzyme with an ion exchange resin.
2. Magnesium, calcium and ferrous ions were without effect in restoring polysaccharide-synthesizing activity to the enzyme.
3. The essential metal for this algal phosphorylase was found to be manganese ion.
4. The logarithm of the formation constant for phosphorylase-manganese was calculated from the activity curve to be 3.6.

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A Chemical Inhibitor of Auxin-Induced Lateral Root Initiation in Roots of *Pisum*

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Lateral root initiation in the roots of seedling peas appears to involve a variety of chemical substances in the seedling, some promotive to the process and others inhibitory. In an earlier study (Torrey 1956) evidence was given for the involvement in lateral root initiation of a number of known plant constituents including indoleacetic acid, thiamin, nicotinic acid, adenine and several trace elements which, under certain conditions, could become limiting factors in the initiation process. In that work, some evidence for the action of inhibitors was presented. Crude ether extracts of pea roots were found to be inhibitory to lateral root initiation. The work discussed here is an extension of the earlier study and centers about our attempts to isolate and characterize the extractable materials in pea roots which are inhibitory to lateral root initiation in the pea plant.

The inhibition of lateral root initiation in seedling roots by an endogenous inhibitor probably produced in the root tip has been suggested by several workers (Thimann 1936, Geissbühler 1953, Nutman 1953, Libbert 1956, Torrey 1956). Indirect evidence for its existence came from studies of root initiation in decapitated roots and experiments with isolated roots grown in culture. The extraction from pea roots of ether-soluble materials which inhibited root initiation in etiolated pea epicotyls was reported by Howell (1954) and by Libbert (1956). That such an inhibitor might act in controlling lateral root initiation was suggested by Libbert (1957). The inhibitory effect of such an extract on root initiation in excised pea root segments was shown by Torrey (1956).

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The presence of endogenous inhibitors of developmental processes in plants has been reported rather frequently in recent years (see review by Bentley 1958). Thus far, relatively little evidence as to the chemical nature of these inhibitors has been obtained. In their postulated function as inhibitors of specific processes in plant development, such substances might play key roles in normal plant development. It is important that more evidence be obtained of their chemical nature and then, more specifically, of their physiological roles.

Material and Methods

1. *Extraction of plant materials.* In all experiments reported here the plant materials extracted were whole roots excised from pea seedlings grown in the light for 7—10 days. Seeds of garden pea, *Pisum sativum*, variety Alaska, were soaked 6 hr. in tap water, then spread in a single layer on cheese cloth over a galvanized wire screen tray supported on a Pyrex dish containing tap water. The seeds were covered with moistened cheese cloth dipping into the water as a wick during the first 72 hr. germination at 25°C. in the dark. Thereafter the cloth over the seeds was removed and the germinator tray and dish moved to a table illuminated at about 400 f.c. with warm white slimline fluorescent lamps. The pea seedlings grew well as normal seedlings and the single tap root of each plant formed numerous branches. No special precautions were taken to darken the roots growing into the Pyrex dish. After 7—10 days the roots were harvested by cutting them off with a razor blade below the cotyledons. After determining the fresh weight, the roots were plunged into liquid nitrogen and the solidified tissues then ground to a fine powder in a Waring blender for about one minute. Usually, batches of about 100 gm. fresh weight were extracted. The powder was washed into a flask with cold absolute ethanol, using about 2—3 times as much alcohol as the volume of the powder. The slurry was stirred vigorously, then stored in the dark at 5°C. for 24—48 hr. The mixture was filtered in a Büchner funnel to remove the plant debris which was then rinsed with fresh alcohol and finally the alcohol was evaporated off in a flash-evaporator under vacuum with warming to 30°C. The aqueous residue was yellow-green in color. A 3 per cent solution of sodium bicarbonate was added to make up a volume of about 200 ml. at about pH 8.7. Peroxide-free diethyl ether of equal volume was added and an ether fraction, referred to as the alkaline fraction, was partitioned in a separatory funnel. The ether fraction was collected and fresh ether added in two successive extractions. The ether extracts were combined and evaporated to dryness at room temperature, then taken up in 1 ml. of fresh ether and stored in a sealed vial in the refrigerator until assay.

To the remaining aqueous fraction was added 5 drops of 0.02 per cent aqueous methyl orange for each 30 ml. of solution, following the method of Larsen (1955 a) and then 1 N HCl was added until a permanent pink color indicated a pH of 3.5. The acidic solution was reextracted three times with fresh ether, the ether fractions combined as the acidic fraction, evaporated to dryness, taken up in 1 ml. fresh ether and stored as above until testing.

2. *Chromatography of plant extracts.* One and one-half by twelve inch strips of Whatman No. 1 paper were cut and usually rinsed in the solvent to be used, then

air dried. A measured volume of the ethereal plant extract varying from 10 to 50 μ l was applied drop wise to a spot 1 $\frac{1}{2}$ inch from the bottom of the strip (Figure 1 B) and allowed to dry. The top of the strip was attached to a hooked glass rod which projected through a large rubber stopper of a Pyrex glass cylinder approximately 3 $\frac{1}{2}$ inches in diameter \times 18 inches high. The solvent was placed in the cylinder to a height of 2 inches and the paper equilibrated above the solvent for about 1 hr. at 25°C. in the dark. Then the paper was lowered into the solvent and allowed to ascend the paper to about nine inches. The paper was removed to dry in a dust-free cabinet with air circulation. Air drying at about 22°C. in the dark was allowed to proceed for 48 hr. before the chromatogram was cut into sections. In handling of the chromatograms after the run, aseptic procedures were always used.

Various solvents were tried during the course of the investigation. The following solvent systems used for auxin chromatography (reviewed by Larsen 1955 b) were tested: *iso*-propanol: water (9:1); *n*-butanol: 28 per cent aqueous NH_3 : water (10:1:1); *isopropanol*: 28 per cent aqueous NH_3 : water (10:1:1). In addition, ethyl acetate saturated with water was found useful. With the former solvents, runs required about 8 hr.; with the last solvent, the run was completed in 1 $\frac{1}{2}$ hr.

After adequate drying the chromatographic strips were carried to a sterile transfer room and with flame-sterilized forceps and scissors were cut in $\frac{3}{4}$ -inch sections, each corresponding to 0.1 R_f units. Each section was in turn cut into three pieces and dropped into a dry sterile 30 mm.-diameter glass vial closed with a cotton plug (Figure 1 C). All alcoholic solvents as well as the ethyl acetate solvent sterilized the paper during the course of chromatography so that no special procedure was necessary to achieve sterile paper strips, but only to maintain sterility after they had dried.

3. *The lateral root initiation assay.* The assay is based on work with excised pea roots published earlier (Torrey 1956). Sterile 5-mm. root tips of 48 hr.-germinated seedlings of the garden pea, *Pisum sativum*, variety Alaska, were excised aseptically and transferred to an agar nutrient medium in 11 cm. Petri dishes with five tips per dish containing 20 ml. medium. The composition of the nutrient medium per liter was as follows: 242 mg. $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$; 42 mg. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 85 mg. KNO_3 ; 61 mg. KCl ; 20 mg. KH_2PO_4 ; 2.5 mg. $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (freshly prepared stock solution); 0.1 mg. thiamin HCl ; 0.5 mg. nicotinic acid; 1.5 mg. H_3BO_3 ; 1.5 mg. $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$; 4.5 mg. $\text{MnSO}_4 \cdot \text{H}_2\text{O}$; 0.25 mg. $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$; 0.04 mg. $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$; 40 gm. sucrose and 7 gm. Difco Bacto-agar. Glass-distilled water was used. The pH of the medium was adjusted to a final pH after autoclaving of 5.0. The medium was autoclaved at 15 pounds per sq. in. for 15 min., then poured into sterile Petri plates. Root tips, grown in the dark at 25°C. for 7 days, reached an average length of 50–60 mm. and were then ready for use in the assay.

The purpose of the assay was to test for substances present in the plant extract but separated by chromatography, which would influence the initiation of lateral roots produced by auxin treatment of root segments. Substances present in the plant extracts might be expected to inhibit lateral root initiation, to stimulate initiation beyond that produced by the auxin treatment, or to have no effect on the initiation process. By excising the root tip and placing standardized root segments on a nutrient medium containing indoleacetic acid (IAA) at an optimum concentration plus other factors essential to lateral root initiation in peas (Torrey 1956), it should be possible to assay for substances which are inhibitory, promotive or ineffective

STEPS IN LATERAL ROOT INITIATION ASSAY

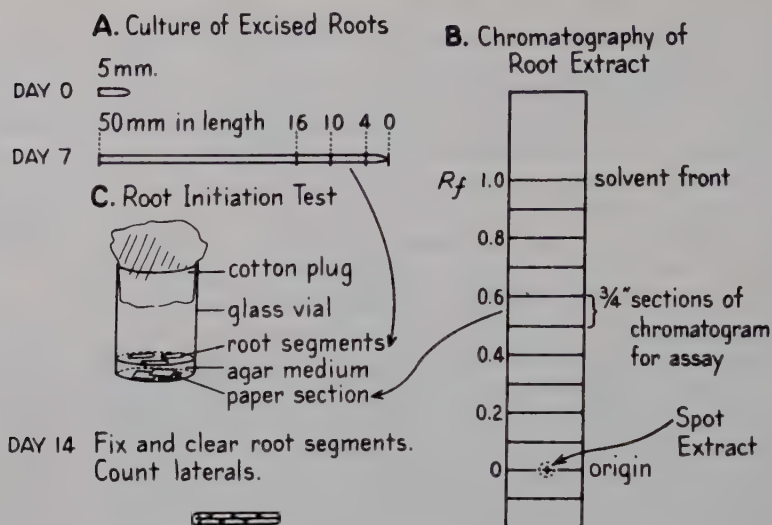


Figure 1. A diagrammatic representation of the steps followed in the lateral root initiation assay. The procedure is described in the text.

on lateral root initiation. Under these conditions, the root segments do not elongate so that the effects can be attributed to the initiation process itself.

The following procedure was adopted. Sterile vials containing cut up sections of the paper chromatogram were numbered in sequence, with any given series of vials representing an entire chromatogram. A paper control was run which included the bottom piece of the chromatogram which had been exposed only to solvents and, in addition, a no-paper control was also set up which contained no chromatogram section. Into each vial was pipetted aseptically 1.5 ml. of warm (liquified) nutrient medium as described above plus 5×10^{-5} M indoleacetic acid and sufficient sterile 0.01 N NaOH to raise the pH of the medium to pH 6.5. The vial was agitated to assure complete wetting and immersion of the chromatogram pieces before solidification of the agar and to accelerate the elution of the substances of plant extract origin from the chromatographic paper section. Usually the vials were prepared several hours to a day before the segments were inserted.

One-week-old pea roots grown in culture were used as the source of root segments. The terminal 4-mm. tip of each root was excised and then two more 6-mm. segments were cut from each root (Figure 1 A). For this test the 4-mm. root tip was discarded and the 6-mm. segments used. The mid-segments produced slightly fewer laterals on the average than the basal-segments, so for each assay series, only segments of one type were used. Ten or twelve segments were transferred to each vial until the entire series was set up. For each set of 30 Petri dishes (150 roots), two chromatographic strips could easily be assayed.

The series of vials was placed in the dark in a high-humidity room (85 per cent relative humidity) at 25°C. for one week. At the end of this time, root initiation was completed. The segments were removed from a vial and were placed in a fixative

(made up of 90 parts 70 per cent ethanol, 5 parts glacial acetic acid, and 5 parts formalin) in a small clean vial, which was aspirated and then stoppered with a cork and labelled. After about 48 hr. the root segments were translucent and root primordia were readily apparent under the dissecting microscope with transmitted light. The number of primordia on each segment was counted, mean values for the segments were determined and the results were plotted as a histogram using R_f as the abscissa and the average number of laterals per ten roots as ordinate. From the no-paper control measurements, the standard error (SE) of the mean was calculated and the fiducial limits at the 5 per cent confidence level (approximately $2.2 \times SE$ in these tests) for each assay were determined and are shown in the histograms. Values greater than or less than these limits are shown in black on the histograms.

4. *The root elongation assay.* It was interesting to compare the effects of plant extracts on lateral root initiation with their effects on root elongation. An assay was devised using an almost identical procedure to that already described in which the excised 4-mm. root tips, rather than root segments, were transferred to the nutrient agar contained in vials into which had been eluted substances separated by chromatography. Ten to twelve tips were placed in each vial in parallel rows and elongation allowed to proceed in the dark at 25°C. and 85 per cent relative humidity for 4 days. Then individual measurements of root length were made with a millimeter ruler and averages determined and plotted as per cent of elongation of the control.

Experimental Results

A. Use of the Assay with Known Compounds

1. *Solvents alone.* In the initial stages of the work, a number of solvent systems were tested to determine those most suited for the separation by paper chromatography of substances in the plant extract active in affecting the process of lateral root initiation. Several of the solvent systems used in auxin chromatography were tested at the outset. Mixtures of *isopropanol* and water, *isopropanol*, ammonia, and water and *n*-butanol, ammonia, and water were tried. All accomplished some separation of biologically-active components of the ethereal root extracts and all were equally desirable so far as the assay was concerned in that, after drying, they left a sterile paper chromatogram. After a number of runs with known compounds and unknown plant extracts in the different solvent systems, a choice was made of the solvent mixture *n*-butanol, 28 per cent aqueous ammonia, and water in the ratio 10 : 1 : 1 for routine testing.

In Figure 2 A is shown a typical histogram from a chromatogram which had been run with the solvents alone and then tested in the lateral root initiation assay. Although there was some variation in the average number of lateral roots initiated in the presence of various sections of the chromatogram, in only one set did it barely reach the level of significance at the 5 per

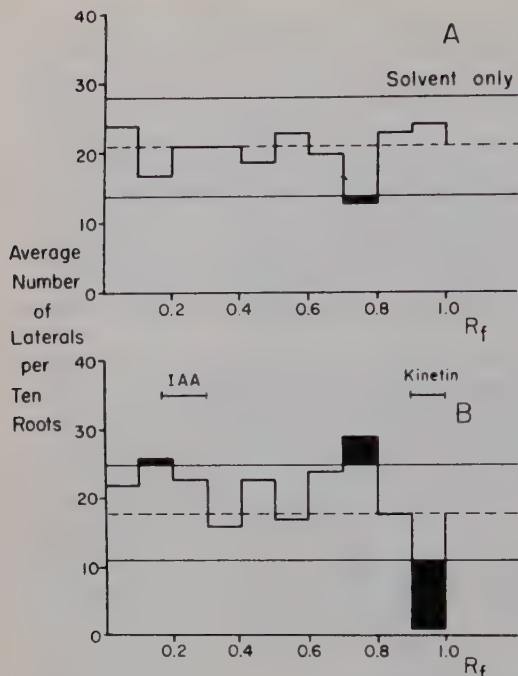


Figure 2. Histograms showing the results of lateral root initiation assay of paper chromatograms. A. Assay of the solvents alone, using *n*-butanol: NH_3 : H_2O (10:1:1). B. Assay of known compounds separated with the butanol-ammonia-water solvent. See text.

cent level. Thus, individual sets in a series appear to give quite reproducible results and there is no evidence of contaminants in the solvent which affect lateral root initiation in any way. Comparable runs with other solvent systems alone showed similar results.

2. *The effects of added IAA and kinetin.* It was interesting to test known compounds which might affect lateral root initiation in the assay system so that they might be used as markers in analyzing the effects of unknown plant extracts. A mixture of IAA and kinetin (6-furfurylamino purine) was prepared, spotted on the chromatogram, and run in the butanol : NH_3 : H_2O solvent, then assayed. In Figure 2 B is shown a histogram from such an experiment in which 10 μl of an absolute ethyl alcohol solution containing 1.02 mg./ml. IAA and 0.15 mg./ml. kinetin was spotted at the origin, run 9 hr. at 25°C. in the dark, then dried and assayed. Parallel strips were run, then sprayed with chemical reagents giving color reactions to allow localization of the knowns by an independent means. The Salkowski reagent of Gordon and Weber (1951) was used to locate IAA and the cysteine — H_2SO_4 reagent of Buchanan (1951) for color identification and location of kinetin. Their positions as determined by color tests are shown above the histogram in Figure 2 B.

In this solvent, IAA ran consistently at about R_f 0.2—0.3 and kinetin ran at the front at R_f 0.9—1.0. In the bioassay, the IAA showed no effect. This result is to be expected, since the assay medium already contained IAA sufficient for optimum lateral root initiation under these conditions. Thus the effect of extracted naturally-occurring IAA present in plant tissues should not be apparent in this assay. Only if another natural auxin, differing in its action from IAA, were extracted and chromatographed would it become apparent in the assay.

Kinetin, on the other hand, was clearly apparent in the bioassay, producing marked inhibition of lateral root initiation and reducing initiation almost to zero. This effect was at a final kinetin concentration in the assay medium, assuming no loss in chromatography, of 1 part per million. The slight stimulation of R_f 0.7—0.8 might be attributable to a contaminant of the kinetin preparation such as adenine (cf. Miller *et al.* 1956), although its R_f in these solvents was not determined. It has already been shown (Torrey 1956) that adenine has a stimulating effect on lateral root initiation in this system.

B. Assays of Pea Root Extracts

1. *Alkaline and Acidic extracts.* In earlier work (Torrey 1956) it had been found that a crude ether extract of pea roots contained substances inhibitory to lateral root initiation. In the present study, an attempt was made to separate the crude extract from pea seedling roots into two fractions. It was found that materials inhibitory to lateral root initiation were extracted from pea roots with diethyl ether, ethyl alcohol and even distilled water. For convenience of manipulation, the cold absolute ethyl alcohol extraction described above was adopted. The subsequent steps involving ether extraction of the alkaline aqueous residue after removal of the alcohol and then acidification and further ether extraction are procedures routinely used for the extraction of auxins (Larsen 1955 b). The alkaline and acidic ether fractions are readily chromatographed and tested separately.

In Figure 3 are shown the results of representative bioassays of the alkaline (Figure 3 A) and acidic fractions (Figure 3 B) after separation by chromatographing with n-butanol, ammonia, water. In both cases 50 μ l of the ether extract was applied to the paper from an extraction of approximately 100 gm. fresh weight of pea roots concentrated to 1 ml. Thus, each run represented extracted material equivalent to about 5 gm. fresh weight of pea roots.

In the alkaline extract (Figure 3 A) are two inhibitory materials, one with R_f 0.2—0.3 and a second spread over a region from R_f 0.6—0.9. The latter material was at such a concentration as to cause complete inhibition of

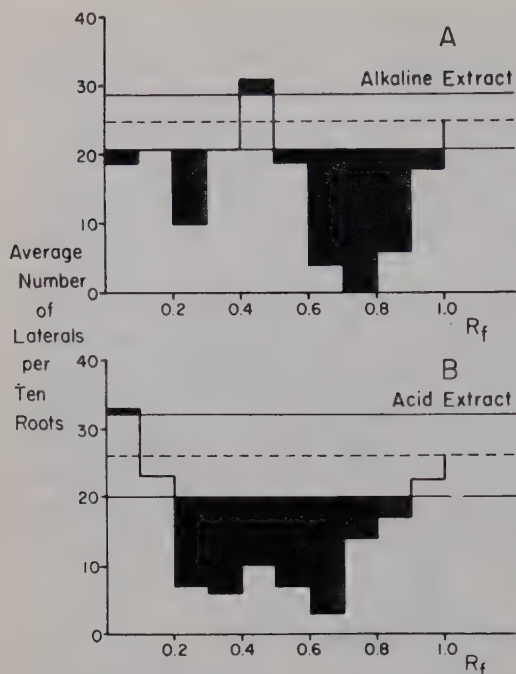


Figure 3. Histograms showing the results of lateral root initiation assay of paper chromatograms of alkaline (A) and acid (B) extracts of pea roots separated with the butanol-ammonia-water solvent system.

lateral root initiation between R_f 0.7—0.8. The slight stimulatory region around R_f 0.5 is of questionable significance.

In the acidic extract (Figure 3 B) a wide band of inhibition was apparent, spreading from R_f 0.2—0.7 and producing inhibition at R_f 0.6—0.7 almost comparable to that of the alkaline extract. It seems apparent that in the total ether extract there are several, probably at least three, substances which cause inhibition of lateral root initiation in the assay. In subsequent work which is here reported, attention was centered upon the alkaline extract in an attempt to achieve some idea of the chemical nature of the inhibitors present.

Using the butanol, ammonia, water solvent system to separate the alkaline extract, it was noted that in the region of the chromatogram producing maximum inhibition of lateral root initiation, *i.e.*, between R_f 0.6 and 0.9, there ran a yellow-colored pigment which corresponded closely to the distribution of the inhibitor, as judged from the bioassay. No UV-fluorescence was present over this region. When sprayed with Salkowski reagent, the yellow color was brightened immediately apparently due to acidity, but the whole region turned a purple-brown color when the chromatogram was incubated at 70°C. for several minutes. It was difficult to judge whether the

Salkowski-positive material was the yellow pigment or was superimposed upon it.

At R_f 0.2—0.3 of the alkaline extract separated with the butanol, ammonia, water solvent, there was no natural pigmentation. Under UV-illumination, there was apparent a bright-blue UV-fluorescence. After spraying with Salkowski reagent, an orange-yellow color was noted on heating and with Ehrlich's reagent, the spot produced a yellow color in white light.

Other solvent systems were tried in an attempt to separate the yellow pigment from the Salkowski-positive material, if possible. This separation was achieved by the use of ethyl acetate saturated with water as the ascending solvent. With this solvent, the yellow pigment remained at the origin, and a clear, slightly oily spot at R_f 0.3—0.6 was found to give a purple-brown color with Salkowski reagent after heating. The blue UV-fluorescent material also remained at the origin in this solvent. In Figure 4 A is shown the bioassay of the alkaline extract chromatographed with the ethyl acetate-water solvent at 23°C. Note that inhibition of lateral root initiation was strongest at R_f 0.4 but also occurred at the origin. Figure 4 B shows that the material which runs at R_f 0.4 in ethyl acetate-water is also markedly inhibiting to root elongation. In butanol : NH_3 : water (10 : 1 : 1) at 25°C. the oily material ran at R_f 0.7—0.8; with butanol : acetic acid : water (4 : 1 : 5) at R_f 0.5—0.6; and with acetic acid : water (15 : 85), the inhibitor stayed at the origin. With the ethyl acetate-water solvent, proper separation of the inhibitor was highly temperature sensitive; the material ran at the front of the solvent at 30°C., at R_f 0.6—0.8 at 26° C. and streaked badly at 5°C.

No further attempt has been made to identify the UV-fluorescent material in the alkaline extract, but an attempt was made to characterize the oily material which was separable with the ethyl acetate solvent system. The total alkaline extract from 100 gm. fresh weight of pea roots was chromatographed on large rectangular sheets with the ethyl acetate-water solvent, the paper at the R_f containing the oily material was eluted, the elute concentrated and then studied in the following tests.

2. *Chemical properties of the alkaline extract inhibitor.* The chromatographed material was readily identified on paper by its oily character producing a translucent spot when the paper was examined in transmitted light. Thus it was easily possible to run a number of tests for chemical reactivity of the material on paper. The compound was quite stable on paper or stored in ether or absolute ethyl alcohol at 5°C. It was soluble in water and could be eluted from paper into an aqueous medium.

Ultraviolet absorption was studied by exposing the paper strip over Kodagraph contact paper to a very brief illumination with ultraviolet light from

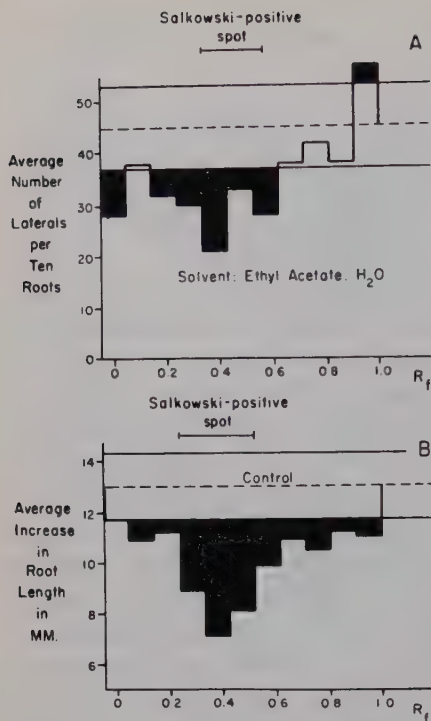


Figure 4. Histograms showing the results of lateral root initiation assay (A) and root elongation assay (B) of the alkaline extract separated by paper chromatography using ethyl acetate saturated with water as solvent.

a germicidal lamp. The inhibitor showed strong ultraviolet absorption, but never any UV-fluorescence alone or after any of the chemical tests.

The unknown showed a green color (pH 4.0–5.6) when the paper was sprayed with an aqueous solution of brom cresol green, indicating its slightly acidic nature.

Although the Salkowski reagent whether made up with H_2SO_4 (Tang and Bonner 1947) or with perchloric acid (Gordon and Weber 1951), produces a purple-brown color, Ehrlich's reagent (1 per cent *p*-dimethylaminobenzaldehyde in 1 *N* HCl, see Larsen 1955 b) showed no color reaction following heating at 65°C. for 10 min. The lack of response to Ehrlich's reagent is strong evidence against the presence of an indole nucleus. The nitrous acid reagent used by Sen and Leopold (1954) (made up of 1 gm. KNO_2 , 20 ml. HNO_3 , 80 ml. 95 per cent ethyl alcohol) produced a yellow color.

The unknown gave no color reaction with ninhydrin (see Consden *et al.* 1944), indicating the absence of amino groups. It likewise gave no color reaction with the naphthoresorcinol-trichloroacetic acid reagent of Partridge and Westwall (1948) which is specific for keto sugars and related compounds.

Several reagents specific for reducing groups gave positive color reactions.

Thus the ammonium molybdate reagent of Aronoff and Vernon (1950) (made up of 20 ml. 10 per cent ammonium molybdate added to 3 ml. concentrated HCl with shaking, then 5 gm. NH_4Cl , sprayed on paper, dried and heated at 70°C . for 20 min.) produced a bright blue color. The benzidine test of Swain (1953) (made up of 0.5 gm. benzidine, 20 ml. glacial acetic acid and 80 ml. absolute ethyl alcohol, sprayed on paper, dried and heated at 105°C . for 15 min.) produced a yellow-brown spot which was unchanged after further spraying with sodium carbonate (20 per cent weight per volume).

The evidence from the above tests suggested that the unknown compound possessed the general properties of a phenolic compound, being weakly acidic and showing reducing properties, more likely attributable to an aldehyde than a keto group. Several tests with reagents reactive with phenolic compounds further strengthened this view. Thus with Millon's test described by Clarke and Nord (1955) (made up of 1 part mercury to 1 part concentrated HNO_3 by weight dissolved in the hood and diluted with 2 parts water by weight; mixed 50 : 50 volume for volume with absolute ethyl alcohol and sprayed) a bright yellow color was produced, suggesting either a mono- or para-disubstituted phenol. With alcoholic iron chloride and HCl (Clarke and Nord 1955) a faint purple color was produced; with aqueous FeCl_3 a faint purple-pink color was formed, suggesting also a phenolic compound. A spray with concentrated H_2SO_4 produced a yellow-orange color characteristic of phenols.

The ultraviolet absorption properties of a sample purified by paper chromatography with the ethyl acetate-water solvent system were determined in absolute ethyl alcoholic solution with a Beckman spectrophotometer. A major absorption peak occurred at 225 $\text{m}\mu$ and a lesser peak at 280 $\text{m}\mu$; absorption dropped off to almost zero at 400 $\text{m}\mu$.

Discussion

The occurrence in plant extracts of inhibitors of auxin-induced processes is best known with respect to the process of shoot and root cell elongation (see review of inhibitors by Audus 1959). Many investigators have reported the inhibitory action of extracts of a variety of plant tissues on *Avena* coleoptile section elongation (see review by Bentley 1958). In some cases, assays using root cell elongation and its inhibition have been reported (*e.g.*, Alexander 1953, Kefford 1955, Audus and Gunning 1958, Pilet 1958). Prominent in discussions of inhibitors extractable from plant tissues is inhibitor β , an ether-soluble acidic substance first designated by Bennet-Clark *et al.* (1952). In the present work, it is not unlikely that the material present in the acid extract which causes marked inhibition of lateral root initiation over a wide

R_1 range corresponds in part at least to inhibitor β . It seems probable from the work of Varga (1957, 1958), Köves (1957) and others that inhibitor β comprises a complex of materials. Further work on the acid extract from pea roots must be carried out before any conclusion can be made as to its chemical nature. Pilet (1958) found a material he called inhibitor β in the acidic extract of Lens roots. He was not able to identify it chemically but implied that it might serve some function in the control of root cell elongation.

The inhibitor studied most carefully in this work is distinct from inhibitor β . From the present evidence, it would appear that the material studied is a specific substance of phenolic nature and not a complex of substances. There is little doubt that other substances which inhibit lateral root initiation also occur in the alkaline extract. The material showing UV-fluorescence and positive Salkowski and Ehrlich's tests might be an indole compound. Further work will be needed to characterize these other substances and to establish the identity of the phenolic inhibitor studied.

In terms of its physical and chemical properties the phenolic inhibitor approaches closely but is not identical with salicylaldehyde. It is at least closely related to this group of phenolic compounds, derived from benzoic acid. Köves (1957) and Varga (1957, 1958) have identified salicylic acid as one of the β -inhibitor complex extracted from oat husks and certain fleshy fruits. Other inhibitors of the complex referred to as β -inhibitor included the closely related phenolic compounds cinnamic acid, *m*- and *p*-oxybenzoic acid and ferulic acid (4-hydroxy-3-methoxycinnamic acid) as well as coumarin, and *o*- and *p*-coumaric acid. Some of these compounds are known to be inhibitors of root elongation (Torrey 1956) and might well be expected to inhibit lateral root initiation also.

It should be noted that the phenolic inhibitor studied here gives a positive Salkowski reaction, which initially suggested that it might be an indole compound. Lack of color reaction to Ehrlich's reagent eliminated this possibility. The lack of specificity of the Salkowski reagent has been pointed out already by Stowe *et al.* (1958) who pointedly listed several phenolic compounds which produce a color when reacted with Salkowski reagent. Under our conditions, catechol produced a bright purple-pink color with Salkowski reagent, salicylic acid a faint purple color, salicylaldehyde a bright red-pink color and methylsalicylate a pale blue color. Since these phenols are likely to be extracted from plant tissues by procedures used for auxins, considerable care must be taken to exclude them from the general category of "unknown indole derivatives". This is particularly important if they possess physiological activity.

The present evidence in no way establishes a physiological role for the alkaline inhibitor in normal root development. But it lends evidence to the

idea of a naturally-occurring substance which interacts with IAA in the determination of the site of lateral root initiation. It is clearly urgent that the chemical identity of these inhibitors, both acidic and alkaline, be established so that the physiological experiments concerning their occurrence, distribution and interaction with IAA and other factors affecting lateral root initiation can have real meaning.

Physiological activity of phenolic substances in plants is usually associated with oxidation systems such as the polyphenoloxidases. Little direct evidence for their involvement in normal growth or development in roots exists. It is interesting, however, that the involvement of some phenolic constituent has been postulated (Pilet and Galston 1955) in the control of root cell elongation by an auxin oxidation system.

Inhibition of lateral root initiation as tested in the assay described above could have at least two causes: 1) a specific inhibition of the auxin-induced cell divisions leading to root primordium formation or 2) the destruction of all IAA activity in the testing medium by an oxidation system, which would thereby remove the prime chemical initiator of the essential cell divisions. The evidence presented above in Figure 4 A and 4 B favors the first interpretation since the inhibitor affects not only root initiation but also causes marked inhibition of root elongation. Such an inhibitor would then be acting in a fashion analogous to that of the antimetabolites reported earlier (Torrey 1956) or like kinetin described above. However, the possibility is not excluded that the inhibitor, separated by chromatography, acts by virtue of its affect on IAA directly. The action *in vitro* of phenolic compounds on IAA oxidation has already been referred to above. Further examination of the assay should establish which type of inhibition is involved in the cases described here.

Summary

Using a new lateral root initiation assay based on the stimulation or inhibition of indoleacetic acid-induced root initiation in excised root segments of *Pisum sativum* cultured *in vitro*, a study has been made of ether- and alcohol-soluble inhibitors extracted from seedling roots of *Pisum* and separated by paper chromatography. Substances with characteristic R_f values in a given solvent system were eluted directly from paper sections into a sterile nutrient medium in which root segments were allowed to develop new root primordia under the influence of added auxin. The detection of an inhibitor was dependent on the degree of suppression of root initiation.

Inhibitors of lateral root initiation were detected in both the alkaline and the acidic ether-soluble fraction of a cold absolute ethanol extract of seedling

pea roots. A detailed study of the alkaline fraction separated by paper chromatography showed that two inhibitor spots were present. The first compound with R_f 0.2—0.3 in an *n*-butanol-ammonia-water solvent showed a bright-blue fluorescence in ultraviolet light, an orange-red color after spraying with Salkowski reagent and a yellow color with Ehrlich's reagent. The compound was not further characterized. A second compound in the alkaline extract, which appeared to be the most active inhibitor, had an R_f of 0.7—0.8 in the same solvent system, showed no UV-fluorescence, formed a purple-brown color with Salkowski reagent but no color with Ehrlich's reagent. When chromatographed at 23°C. with water-saturated ethyl acetate as the solvent, the compound was apparent as an oily spot at R_f 0.3—0.6. From its solubilities, its slightly acidic nature, its reducing properties, its reaction to a variety of color tests, and its ultraviolet absorption characteristics, the inhibitor has been tentatively identified as a phenolic compound. Its possible physiological role in controlling the site of lateral root initiation in pea seedlings has been suggested, but elucidation of its role awaits further physiological study.

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Penetration and Stability of GS-1 in Plant Tissue

By

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Introduction

GS-1, 2-(1'oxy-2-pyridylthio) imidazoline hydrobromide,¹ shows a high degree of activity against plant pathogenic fungi and bacteria *in vitro* and is active against several parasites on plants and seeds (1). Systemic activity in plants as well as in seeds has been reported (2). One of the requirements for a successful chemotherapeutant is that its activity, after penetration into the plant tissue, should not disappear by either metabolic or other processes before it can act against the parasites present in this tissue. The purpose of this study was to test the fungicidal activity of GS-1 with respect to this requirement.

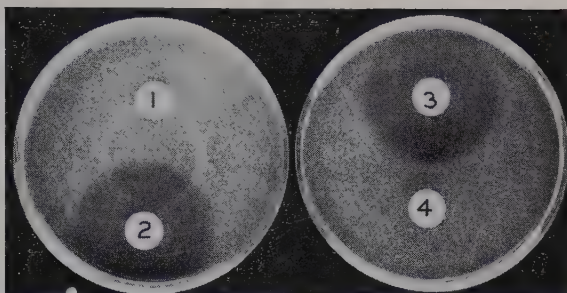
Methods

Bioassaying was performed on plates of potato dextrose agar which were flooded with a spore suspension of *Glomerella cingulata*, (Stonem.) Spauld. & Schrenk, (density 35 on a Klett-Summerson spectrophotometer), and dried for 20 minutes at 40°C. Filter paper discs (S & S No. 740 E), 12.7 mm. in diameter, were saturated with the liquid to be tested and placed on the seeded agar plates. Reading of the zones of inhibition was made after 24 hours. Orange rind, 8 mm. in diameter, and seeds were bioassayed by placing them on the *Glomerella cingulata* seeded agar plates.

¹ From Chas. Pfizer & Co., Inc., Brooklyn 6, N.Y. Patent applied for.

Figure 1. *Inactivation of GS-1, 10 p.p.m., after addition to juice expressed from leaves of bean plants, var. Pinto; no inactivation when pH of juice was lowered to pH 4.*

1. GS-1 in juice, pH 5.7
2. GS-1 in juice, pH 4.0
3. GS-1 in water
4. Juice, pH 4 (no GS-1).



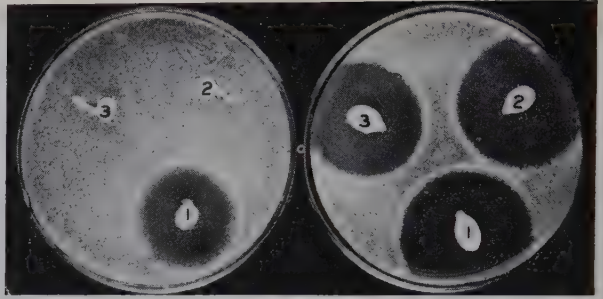
Results

When bean plants, *Phaseolus vulgaris* L., (Pinto variety) were placed with their roots in a solution containing 200 p.p.m. or more of GS-1 for 24 hours, large inhibition zones were obtained when the juice, expressed from the leaves, was bioassayed. No inhibition zones appeared at concentrations of 100 p.p.m. or lower. In experiments *in vitro*, in which GS-1 was added to the expressed juice, it was demonstrated that low concentrations of GS-1, such as 20 p.p.m., were inactivated by the juice of bean leaves. The inactivation was prevented when the juice was lowered to pH 4 by a citrate-phosphate buffer before addition of the GS-1 (Figure 1). The GS-1 inactivating principle in juice of bean leaves does not work at a low pH. Similar results were obtained with juice from cucumber leaves, but GS-1 maintained its activity in untreated juice from *Oxalis corniculata* L., (pH 1.9).

The penetration and stability of GS-1 was studied in the following seeds: *Pisum sativum* L. (varieties Chancellor and Early Perfection), *Phaseolus vulgaris* L. (var. Pinto), *Vigna sinensis* Endl., *Cucumis sativus* L. (Marketer variety), *Cucurbita Pepo* L. (var. Danish squash), and *Zea Mays* L. After soaking these seeds for 24 hours in an aqueous solution of GS-1 containing 100 p.p.m. and rinsing in tap water, the seed coats were removed and the cotyledons bioassayed. All showed significant to large inhibition zones. This demonstrated the penetration of GS-1 through the seed coat into the interior of the seeds. However, when the seeds, after the treatment with GS-1, were kept for 10 to 48 hours in a moist environment and subsequently bioassayed, all fungicidal activity had disappeared from the leguminous seeds, but not, or at least to a lesser extent, from the other seeds. Figure 2 gives the results with pea and squash seeds.

A study was made of the GS-1 penetration of the rind of oranges, *Citrus sinensis* Osbeck. Untreated Valencia oranges, kindly provided by Dr. L. J. Klotz, Citrus Experiment Station, Riverside, California, were used for the

Figure 2. *Inactivation of GS-1 in pea seeds (var. Chancellor), left; no inactivation in seeds of Danish squash, Cucurbita Pepo L., right.* Seeds were soaked in GS-1, 100 p.p.m., for 24 hours, rinsed with tap water, and bioassayed (1) immediately, (2) after 24 hours, and (3) after 48 hours.



experiments. When the oranges, or detached rind, were dipped in a GS-1 solution (1000 p.p.m.) for 4 minutes and washed with tap water after the treatment, no fungicidal activity was detected by bioassaying. However, when the GS-1 solution in which the detached rind was dipped was heated to 60°C., or when the detached rind, before or after the treatment with the GS-1, was heated by hot air (100—175°C.) for two minutes, a large inhibition zone was obtained on bioassaying, while no inhibition zones were present after heating without the GS-1 (Figure 3).

After dipping a whole orange in a GS-1 solution (1000 p.p.m.) for four minutes and the subsequent heating of the detached rind by hot air, the presence of GS-1 could be demonstrated in the rind, even when the thin outer layer which had been in direct contact with the solution was removed before bioassaying. This demonstrates that GS-1 penetrates oranges.

In order to get more information on the nature of the GS-1 inhibiting principle in the orange rind, experiments were carried out during which it was found that by a simple soaking of the rind in water for one hour or longer the activity of the inhibitor was diminished to such an extent that GS-1 could be demonstrated in it after a four-minute dip, while without the presoaking in water no inhibition zones were obtained. Similar results were

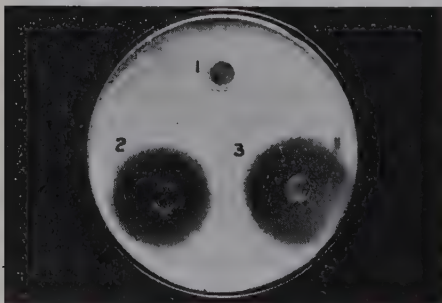


Figure 3. *Protection of GS-1 from inactivation in orange rind by heat treatment.* Detached orange rind was dipped in GS-1, 1000 p.p.m., for 4 minutes and discs from the flavedo part of the rind were bioassayed.

1. Rind dipped in unheated solution.
2. Rind dipped in solution at 60°C.
3. Rind dipped in solution and thereafter heated with hot air (175°C) for two minutes.

obtained when, before treatment with GS-1, the detached rind was exposed to cyanide gas, or dried overnight in an oven at 40°C., or dipped for 10 minutes in alcohol (75 %) or acetone. In the following experiment detached rind was treated as indicated hereafter, and the flavedo part of it subsequently dipped in GS-1 (50 p.p.m.) for four minutes, thoroughly washed with tap water, and bioassayed. Inhibition zones produced by discs of the rind, 8 mm. in diameter, were as follows: After exposure to cyanide gas equivalent to 105 ml. liquid HCN per 100 cu. feet for one hour, 26 mm., after soaking 5 hours in water, 22 mm., after drying in an oven at 40°C., 30 mm., after dipping in alcohol (75 %) for 10 minutes, 29 mm., after dipping in acetone for 10 minutes, 19 mm., and no inhibition zone without pretreatment.

When the flavedo part of the rind of one orange was ground in a Waring blender with 30 ml. of a phosphate buffer solution of pH 7 (0.2 M) and centrifuged for 15 minutes at 5000 r.p.m., the supernatant fluid possessed GS-1 inactivating properties. After lowering the pH of this supernatant to 4 by adding citrate-phosphate buffer to it, no inactivation of GS-1 took place.

The data from these experiments suggest the enzymatic nature of the GS-1 inhibiting principle in orange rind and point in the direction of oxidases. The presence of enzymes resembling peroxidase and cytochrome oxidase in the flavedo part of the orange rind has been reported by Hussein (3). Experiments were carried out with peroxidase, catalase, cytochrome C, and acetone washed pig heart powder, containing cytochrome oxidase. From the last material an extract was prepared by grinding 500 mg. of it in 10 ml. of a KH_2PO_4 - Na_2HPO_4 buffer of pH 7 and centrifuging it for 15 minutes at 5000 r.p.m. Only the supernatant fluid was used. The enzymes were added to an aqueous solution of GS-1, containing 3 p.p.m. Bioassaying was performed after 24 hours. The inhibition zones obtained were as follows: GS-1 alone, 25 mm., GS-1+catalase, 10 p.p.m., 24 mm., GS-1+cytochrome C, 20 p.p.m., 25 mm., GS-1+peroxidase, 10 p.p.m., no inhibition zone and no inhibition zone in GS-1+pig heart extract.

This experiment indicates that peroxidase and cytochrome oxidase which are both common in all kinds of plant material may play a role in the inactivation of GS-1 after it penetrates the tissue. Since in the case of oranges the inhibitor is already eliminated by heating to 135°F. and peroxidase is less sensitive to high temperature, it is suggestive that in the orange rind cytochrome oxidase may be involved in the inactivation of GS-1.

The GS-1 inhibitor in pea seeds was inactivated by dipping the seeds for 20 minutes in water at 60°C. and by an eight-hour dip in an aqueous KCN solution (1000 p.p.m.) or alcohol (75 %) and might therefore be similar or at least closely related to the GS-1 inhibitor in the orange rind.

Inactivation of the GS-1 inhibitor in juice of cucumber leaves was accom-

plished not only by low pH but also dipping the leaves for 20 minutes in KCN (1000 p.p.m.) or alcohol (75 %) but not by heating the leaves to 60°C. A temperature of 100° C. was required. Whether in this case a more heat-stable enzyme, such as peroxidase, could be involved has not been determined.

Discussion

GS-1 is rather stable in some plant tissues but inactivated in others. However, even in the last case, chemotherapeutic activity cannot be excluded *a priori*. In the case of the treatment of peas with GS-1, *e.g.*, it has been shown that the inactivation is a rather slow process, taking at least 10 hours. If an internal parasite is to be killed within this period of time, a good internal disinfection can be accomplished in spite of the eventual disappearance of the antimicrobial agent.

It is presumed that the inactivation of GS-1 is of the oxidation-reduction type since cytochrome oxidase belongs to a chain of enzymes, involved in oxidation reduction reactions.

Summary

GS-1 can be taken up by the roots of beans and cucumbers and transported to the leaves. It penetrates readily into various seeds during a 24-hour soak in a 100 p.p.m. aqueous solution, and into orange rind from a 1000 p.p.m. aqueous solution after a four minute dip. However, inactivation of GS-1 takes place rapidly in expressed juice of bean and cucumber leaves, in bean and pea seeds, and in the flavedo part of the orange rind, while no or much less inactivation occurs in cucurbit and corn seeds and in *Oxalis corniculata* L. The inactivation is believed to be of the oxidation reduction type in which certain plant enzymes are involved, in the case of orange rind probably cytochrome oxidase.

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Carbon Dioxide Acceptor I and II in Photosynthesis of *Polytrichum attenuatum*

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1. Introduction

When photosynthetic rate is plotted as a function of time, the resulting curve is the so-called photosynthesis time curve. The rate of photosynthesis may be measured either by the uptake of carbon dioxide (the CO_2 time curve) or by the output of oxygen per time unit (the O_2 time curve). In previous experiments with the moss species *Polytrichum attenuatum*, the CO_2 time curve has been shown to form a peak after about 1 minute of illumination (cf. Vejlby 1958 a, b); this peak is referred to as the 1 minute peak. It has also been shown that the O_2 time curve of *Polytrichum* does not show any 1 minute peak (Vejlby 1959 a); hence it is reasonable to assume that the peak is representing a light induced uptake of carbon dioxide by a special acceptor, here referred to as acceptor I, the effect of which is the one first observed after the onset of illumination. Under normal conditions the carbon dioxide is subsequently transferred from this acceptor to the usual one, probably ribulose diphosphate, which we shall call acceptor II. This transfer appears to take place at 14° within the first 6 minutes of photosynthesis (Vejlby 1958 b).

If the 1 minute peak represents a CO_2 uptake different from that represented by other parts of the time curve, it may be expected to change in a way different from these other parts when the plants are subjected to various external factors. That this is indeed the case has been demonstrated by Massini (1957), who varied the light intensity, the quality of the light, and

the concentration of carbon dioxide, and who also studied the effects of various chemical substances. The effects of changes in temperature and of intermittent illumination have been studied by van der Veen (1949) and Vejlby (1958 a, b). Whereas Massini used leaf segments of *Datura* and van der Veen used segments of Tobacco leaves and needles of *Sciadopitys verticillata*, the present author worked with species of moss. These plants are particularly suited to the diaferometer method, as the possibility of complications due to variations in the degree of opening of stomata is absent. The present study is concerned with the effect of various factors on the photosynthesis time curve of *Polytrichum attenuatum*, studied in an attempt to isolate, if possible, the part of carbon dioxide uptake represented by the peak in the time curve. The external factors include a special heat treatment of the sample plants, *i.e.*, submersion in water of 45.5 to 50.0°C for a period of 3 minutes, and treatment with a number of enzyme inhibitors and activators.

2. Material, methods, and results

The plants used for the experiments (*Polytrichum attenuatum* Menz.) collected in the Tisvilde Plantation, were stored in an air thermostat at 5 to 10°C as previously described (Vejlby 1958 b, 1959 a). The photosynthetic rate was measured as the uptake of carbon dioxide determined by means of the diaferometer method (Vejlby 1958 a), the rate of the gas stream being 5 to 6 l/h and the temperature of the gas being 15° where not otherwise specified. The light intensity was 255 cal/dm²h (λ 400 to 700 m μ) \sim 8900 lux (conversion according to Gabrielsen 1948). All experiments with exception of those represented by curve *U*, Figure 7, (experiments in 20 % CO₂) were carried out in atmospheric air enriched by addition of 3 % carbon dioxide.

Photosynthesis time curves were drawn for untreated plants as well as for plants which had been submerged in water or in aqueous solutions of various chemicals for shorter or longer periods of time prior to the experiments. In certain cases it seemed difficult to induce reactions in the moss plants merely by submerging them in the solutions mentioned. In order to ensure a proper penetration into the plant tissues a preliminary air-drying at room temperature for 5 to 15 minutes before submerging was included in the method. It should be emphasized that this process of drying is not a very easily definable or reproducible technique. It would undoubtedly be more advantageous to dry the plants at a well defined vapour pressure. This, however is an extremely time consuming procedure (Anderson, Hertz, and Rufelt 1954, Allerup 1959), which is why we used the described simple air-drying technique exclusively for the present study.

1. *Experiments concerning drying and subsequent water uptake.* The effect of a preliminary drying of the plant material upon the photosynthesis time curve appears from a comparison of curve *A* and *B* in Figure 1. Curve *A* represents untreated plants and curve *B* plants which have been dried for 15 minutes and subsequently submerged in water until the initial state of

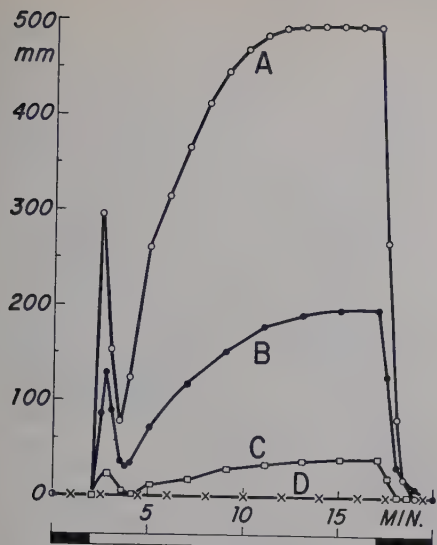


Figure 1.

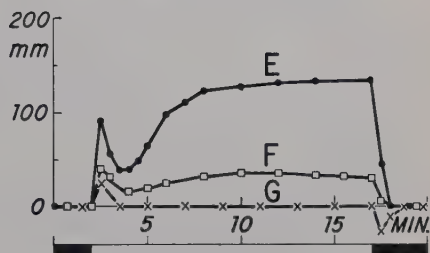


Figure 2.

Figure 1. Photosynthesis time curves for *Polytrichum attenuatum* in 3 % CO_2 measured at 15°C . 5 specimens were used for each measurement (about 0.25 g of fresh weight). A: Untreated plants. B, C and D: plants air-dried at room temperature for 15 minutes and subsequently immersed for 30 minutes in water (B) or in 10^{-2} M 2,4-dinitrophenol for 20 minutes (C) or 30 minutes (D). The temperature of the liquids used for immersion was 20° . Abscissa: time in minutes. Ordinate: galvanometer reading in mm. Black and white areas under the curves indicate dark and light periods, respectively.

Figure 2. Photosynthesis time curves for *P. attenuatum* in 3 % CO_2 measured at 15°C . Prior to the measurements the plants were submerged in phenylurethane solution. E: 10^{-3} M for 30 minutes at room temperature. F: 10^{-2} M for 5 minutes at 30.0°C . G: 10^{-2} M for 5 minutes at 35.0° .

turgidity is reestablished. It appears that a short period of drying preliminary to the experiment causes an inhibition in fully turgid plants which is of approximately the same order of magnitude for the 1 minute peak as for the part of the curve representing steady state photosynthesis.

2. *Experiments with 2,4-dinitrophenol.* If the plants initially are treated as above, but a 20 minute treatment of 10^{-2} M 2,4-dinitrophenol is substituted for the water treatment, a reduction occurs of the initial uptake of carbon dioxide (the 1 minute peak) as well as of the level of the steady state photosynthesis (Figure 1, curve C). The reduction is much more pronounced than that in the case of the water treated plants (curve B). An increase of the period of the dinitrophenol treatment from 20 to 30 minutes causes complete absence of any form of light induced uptake of carbon dioxide (curve D).

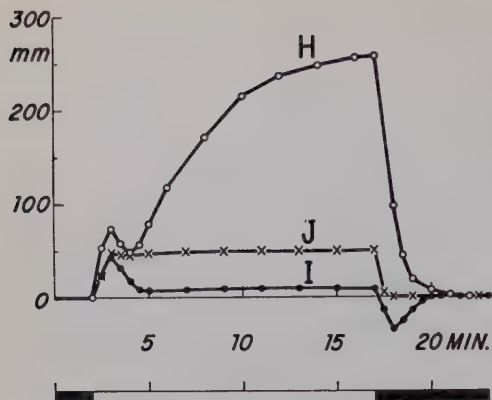


Figure 3.

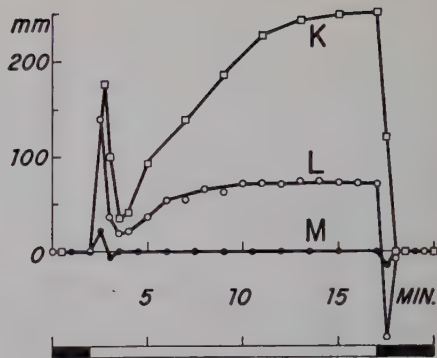


Figure 4.

Figure 3. *Photosynthesis time curves for P. attenuatum in 3 % CO₂. H: Untreated plants measured at 15°C. I: Plants submerged in $5 \cdot 10^{-2}$ M iodoacetamide for 1 hour at room temperature. The curve measured at 15°. J: Same plants as I, but measurements made at 30.5°.*

Figure 4. *Photosynthesis time curve for P. attenuatum in 3 % CO₂ measured at 15°C. Prior to the measurements the plants had been submerged in water for the following periods and at the following temperatures. K: 5 minutes, 35.0°. L: 3 minutes, 45.5°. M: 3 minutes, 50.0°.*

3. *Experiments with phenylurethane.* The results of a 30 minute treatment with 10^{-3} M phenylurethane at room temperature appear in Figure 2, curve E. Due to the impossibility of making solutions of phenylurethane of very much higher concentrations at room temperature, the plants were treated at 30° for 5 minutes with a 10^{-2} M solution (curve F). Compared with the curve for untreated plants (A in Figure 1) curves E as well as F show increasing inhibition of all parts of the time curve with increasing concentrations of phenylurethane. If, however, the same concentration of phenylurethane and the same duration of treatment are used as in the case of curve F, whereas the temperature is raised to 35°, the course of the time curve changes radically (curve G). As compared to curve F the peak is further reduced; at the same time the photosynthetic activity normally following immediately after the occurrence of the 1 minute peak is nonoccurrent. In addition, a new phenomenon (as compared to the curves A, B, C, D, E, and F of Figures 1 and 2) is registered, i.e., a gush of CO₂ occurs immediately when the light is switched off, and the size of the gush corresponds to the amount of carbon dioxide registered as taken up by acceptor I during the formation of the 1 minute peak.

4. *Experiments with iodoacetamide.* It has proved possible to induce the

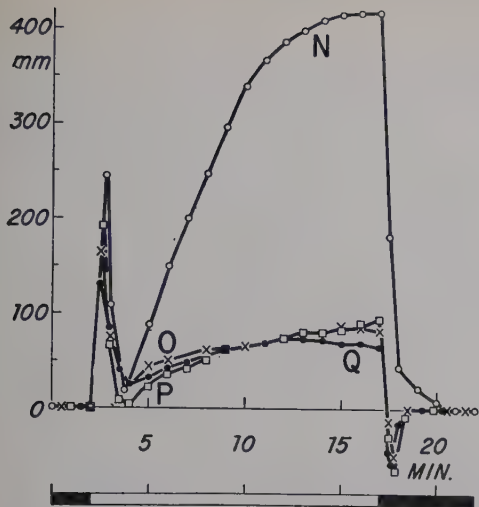


Figure 5.

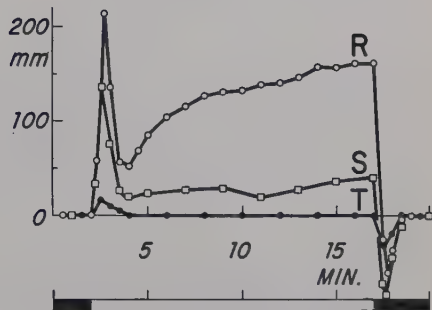


Figure 6.

Figure 5. Photosynthesis time curves for *P. attenuatum* in 3 % CO_2 measured at 15°C . Prior to the measurements the plants had been either untreated (N) or submerged in solutions of KCN at room temperature. O: 10^{-2} M KCN for 30 minutes. P: 10^{-1} M KCN for 20 minutes. Q: $5 \cdot 10^{-1}$ M KCN for 30 minutes.

Figure 6. Photosynthesis time curve for *P. attenuatum* in 3 % CO_2 measured at 15°C . Prior to the measurements the plants had been submerged in $5 \cdot 10^{-1}$ M KCN at room temperature. R: Submersion period 10 minutes. S: Plants dried for 5 minutes at room temperature and subsequently immersed for 10 minutes. T: Plants dried for 10 minutes at room temperature and subsequently immersed for 30 minutes.

occurrence of a similar gush of CO_2 by various other methods. Thus, if the moss plants are submerged for 1 hour in a $5 \cdot 10^{-2}$ M solution of iodoacetamide, the phenomenon shows up distinctly in the time curve (Figure 3, curve I).

The 1 minute peak which is registered at 15° in the time curve of untreated plants has previously been shown to disappear when the experiments are carried out at 30° (Vejlby 1958 a). If a similar experiment is made with the iodoacetamide treated plants, a time curve such as curve J of Figure 3 is obtained. Here, too, the peak disappears, the general photosynthetic activity increases in comparison with that of identically treated plants in curve I due to the higher temperature, and a CO_2 gush can no longer be registered when the light is switched off. Hence the occurrence of the CO_2 gush appears to depend upon the previous uptake by acceptor I of a corresponding amount of carbon dioxide. However, the possibility of inducing a CO_2 gush exists only as long as CO_2 still does adhere to acceptor I.

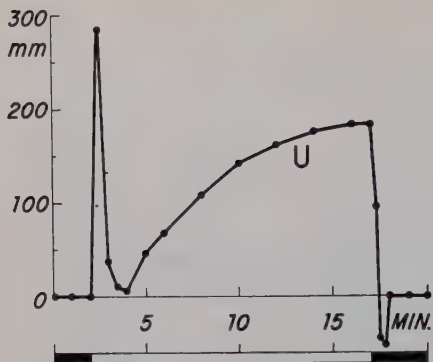


Figure 7. Photosynthesis time curves for *Polytrichum attenuatum* in 20 % CO_2 measured at 15°C .

5. *Experiments with moss plants given heat treatment prior to the photosynthesis measurements.* Van der Veen (1949) has shown previously, in experiments with needles of *Sciadopitys verticillata*, that after being heated for 3 minutes at 51° they were no longer able to show any signs of photosynthesis with the exception of the initial CO_2 uptake corresponding to the 1 minute peak. The amount of carbon dioxide taken up by acceptor I during illumination is retained until the onset of the dark period, and is then released as a gush. This phenomenon is apparently very similar to the one represented by Figure 2, curve G and Figure 3, curve I. In order to decide whether the above heat effect would occur in *Polytrichum* as well, a number of experiments were made submerging the moss plants in water for 5 minutes at 35.0° and for 3 minutes at 45.5° and at 50.0° . The results appear in Figure 4, curves K, L, and M. It is seen that only at the two highest temperatures used was it possible to register a CO_2 gush during the dark period terminating the experiments.

6. *Experiments with cyanide.* Submerging the moss plants in a solution of KCN for 20 to 30 minutes appears to induce the same effect whether the molar concentration is 10^{-2} , 10^{-1} or $5 \cdot 10^{-1}$ (Figure 5, curve O, P, Q). A certain degree of inhibition of the reactions causing the occurrence of the 1 minute peak is found to take place, as is a somewhat more extensive, but surprisingly uniform inhibition of the steady state photosynthesis. In all cases a CO_2 gush was registered after the light was turned off. As apparently, under the given conditions, the effect of the KCN is fairly independent of the concentration used, experiments were made in which the plants were air-dried at room temperature prior to being submerged in a $5 \cdot 10^{-1}$ M solution of KCN (Figure 6). For comparison, a preliminary experiment was made, treating the plants for 10 minutes without previous drying (curve R). If the plants are dried for 5 minutes before being submerged for 10 minutes in the

cyanide solution the effect on steady state photosynthesis is found to be pronounced (curve S). The effect becomes even more evident when the drying period is extended to 10 minutes and the submerging period to 30 minutes, nothing but the peak and the CO_2 gush remaining in this case (curve T).

7. *Experiments with high concentrations of CO_2 .* In the previous paper (Gabrielsen and Vejlbj 1959) the rate of photosynthesis in leaves of potato and beet plants has been shown to be inhibited strongly when the leaves were placed in atmospheric air, to which had been added 20 per cent by volumen of carbon dioxide. The photosynthesis time curve for *Polytrichum* in 20 % carbon dioxide (Figure 7) shows the peak to be strongly increased as might be expected (cf. Massini 1957, Vejlbj 1958 a), whereas the steady state photosynthesis is inhibited. In this case, too, does a CO_2 gush occur when the light is switched off. If the light period, however, is extended to 25 minutes it is no longer possible to register any gush in the following dark period.

8. *Experiments with sulphanilamide.* Sulphanilamide acts as a specific inhibitor for the enzyme carbonic anhydrase, which may possibly play a part during the induction phase of photosynthesis. Experiments were made with *Polytrichum* plants which were either air-dried for 10 minutes and then submerged in a 10^{-2} M solution of sulphanilamide for 10 minutes, or were dried for 20 minutes and submerged for another 20 minutes in the sulphanilamide solution. However, the photosynthesis time curves for plants treated with sulphanilamide were found to be identical with those for plants treated with pure water, and hence sulphanilamide appears to be without effect on the photosynthesis of *Polytrichum attenuatum*.

9 and 10. *Experiments with cysteine and with ethylenediamine tetraacetic acid (EDTA).* In a study of the carboxylation enzyme (diphosphoribulose carboxylase) which catalyzes the formation of phosphoglyceric acid from ribulose diphosphate and carbon dioxide, Weissbach, Horecker and Hurwitz (1956) found that in order to display its full activity this enzyme requires the presence of divalent metal ions, mainly Mg^{++} or Ni^{++} , sulphhydryl compounds, or chelating agents. Therefore, experiments were made treating *Polytrichum* plants with solutions of cysteine and EDTA in order to establish a possible effect of these compounds on the induction phase. The plants were air-dried for 10 minutes and subsequently submerged for 10 minutes in a 10^{-2} M solution of cysteine or of EDTA, equivalent amount of KHCO_3 having been added beforehand to both substances to neutralize the acid groups. In neither case was it possible to demonstrate any effect on the photosynthesis of the moss plants.

11 and 12. *Experiments with arsenate and phosphate.* In the above paper Weissbach *et al.* report the carboxylation enzyme to be inhibited by relatively low concentrations of phosphate and arsenate ions. They state that an

arsenate concentration of 0.02 *M* gives complete inhibition, and that phosphate concentrations of 0.03 *M* and 0.01 *M* give inhibitions of 90 and 70 %, respectively. In the present study, therefore, experiments were made submerging *Polytrichum* plants in an 0.1 *M* NaHAsO₄ solution for 20 and 35 minutes following 5 minutes of air-drying, and in a 0.03 *M* phosphate solution for 20 minutes following 15 minutes of air-drying. The phosphate solution was prepared by mixing 90 ml of a 0.03 *M* solution of Na₂HPO₄ with 10 ml of a 0.03 *M* solution of KH₂PO₄ (pH=7.7).

The reaction of the arsenate-treated plants was similar to that of cyanide-treated ones, the time curve showing some inhibition of the peak and a somewhat higher degree of inhibition of the steady state photosynthesis. In this case, too, a CO₂ gush appeared upon the switching off of the light.

No effects, however, could be demonstrated in the plants treated with phosphate solution. Here the photosynthesis time curve corresponded exactly to that drawn for plants submerged in pure water.

All of the experiments described here were repeated several times. Hence the curves presented in Figures 1 to 7 may be considered to be representative. A survey of the experimental results are found in Table 1.

3. Discussion

In a previous paper (Vejlby 1958 b) it was demonstrated that when *Polytrichum* plants in 3 % carbon dioxide are illuminated for 1/4 to 4 minutes a gush of CO₂ may be registered during the following dark period. If, on the other hand, the duration of the light period is extended to 6 minutes or more, no gush occurs. It appears from the present study that a similar CO₂ gush may be induced in moss plants which have either been 1) exposed to a short period of heating (45 to 50° for 3 minutes) prior to the photosynthesis measurements, 2) treated with iodoacetamide, cyanide or arsenate, or 3) have been fed atmospheric air containing 20 % carbon dioxide during the measurements proper. In all these cases except the last one the duration of the light period seems to be of no importance, the CO₂ gush occurring during the terminating dark period whether the duration of the light period is 3 or 30 minutes. After treatment with phenylurethane the gush could be demonstrated only if the treatment had taken place at high temperatures (35°C), and after treatment with 2,4-dinitrophenol no CO₂ gush whatsoever could be registered.

It thus appears reasonable to establish as a working hypothesis that immediately when the plants are exposed to light, carbon dioxide is taken up by a special acceptor which we call acceptor I. If the light is switched

off within the first 4 minutes of illumination, a smaller or larger fraction of the carbon dioxide thus taken up is given off again. If, however, the light period exceeds 6 minutes, all of the carbon dioxide initially taken up has been transferred to the photosynthetic process proper (cf. Vejlby 1959 a). If the latter is inhibited this will in a number of cases result in the appearance of a CO₂ gush when the light is switched off. In other words, it has been impossible to transfer the carbon dioxide taken up by acceptor I to the assimilation processes. Not every type of inhibition of photosynthesis, however, does cause the said CO₂ gush during the terminating dark period. Thus, in the experiments with 2,4-dinitrophenol, all parts of the time curve appear to be proportionally inhibited with increasing effects of the substance until complete cessation of any form of light induced CO₂ uptake (Figure 1). Likewise, only in a very special case did we succeed in inducing the CO₂ gush in plants treated with phenylurethane (Figure 2, curve G). In the case of iodoacetamide, it proved possible to demonstrate the gush at normal temperature in *Polytrichum attenuatum* (Figure 3, curve I), whereas in a previous experiment with the moss species *Thuidium tamariscinum* we had failed to do so (Vejlby 1959 b, Figure 5); in this latter case, however, the primary peak of the time curve is relatively small, which might account for the absence of a gush. Also, the iodoacetamide concentration was only half that used in the present study, and the duration of the treatment only $\frac{1}{6}$ of the one used here.

Thus, cyanide, arsenate and iodoacetamide appear in addition to their well-known inhibiting effect on photosynthetic rate as such to have a specific inhibiting effect on the process of transfer of the carbon dioxide retained in acceptor I to photosynthesis proper. It is quite conceivable that this effect is centered on the carboxylation enzyme catalyzing the process (diphosphoribulose carboxylase)



(Quayle, Fuller, Benson, and Calvin 1954, cf. Dixon and Webb 1958). The enzyme in question has been studied in detail by Weissbach, Horecker, and Hurwitz (1956), who report that it is inhibited by, among other things, arsenate and phosphate ions. It appears from Table 1, however, that these ions affect the photosynthesis of *Polytrichum* differently, arsenate by causing a distinct inhibition of the induction peak as well as of the steady state photosynthesis and further by inducing a CO₂ gush during the ensuing dark period, while phosphate appears to be of no registerable effect whatsoever. Weissbach *et al.* further report that the carboxylation enzyme is activated by sulphhydryl compounds such as cysteine and by chelating agents such as

Table 1. *Effects of different treatments on the induction phase of photosynthesis.*
I: immersion before photosynthesis experiments, *E*: treatment during experiments.

Treatment of plant material	Preliminary drying	Temp. C°		Inhibition		CO ₂ gush in terminating dark period	Figure
		during immersion	during photosynthesis	peak	steady state		
1. <i>I</i> in water	+	20	15	+	+	—	1 B
2. <i>I</i> in 2,4-dinitrophenol	+	20	15	+	+	—	1 C, D
3. <i>I</i> in phenylurethane <i>M</i> : 10 ⁻³	—	20	15	+	+	—	2 E
<i>M</i> : 10 ⁻²	—	30	15	+	+	—	2 F
<i>M</i> : 10 ⁻²	—	35	15	+	+	+	2 G
4. <i>I</i> in iodoacetamide <i>M</i> : 5 · 10 ⁻²	—	20	15	+	+	+	3 I
<i>M</i> : 5 · 10 ⁻²	—	20	30.5	+	+	—	3 J
5. <i>I</i> in hot water	—	35.0	15	+	+	—	4 K
.....	—	45.5	15	+	+	+	4 L
.....	—	50.0	15	+	+	+	4 M
6. <i>I</i> in cyanide <i>M</i> : 10 ⁻² , 10 ⁻¹ , 5 · 10 ⁻¹	—	20	15	+	+	+	5 O, P, Q
<i>M</i> : 5 · 10 ⁻¹	—	20	15	+	+	+	6 R
.....	+	20	15	+	+	+	6 S, T
7. <i>E</i> in atmospheric air + 20 vol % carbon dioxide	—		15	—	+	+ ²	7 U
8. <i>I</i> in sulphanilamide <i>M</i> : 10 ⁻²	+	20	15	— ¹	— ¹	— ¹	
9. <i>I</i> in cysteine <i>M</i> : 10 ⁻²	+	20	15	— ¹	— ¹	— ¹	
10. <i>I</i> in ethylenediamine tetraacetic acid (EDTA) <i>M</i> : 10 ⁻²	+	20	15	— ¹	— ¹	— ¹	
11. <i>I</i> in arsenate <i>M</i> : 10 ⁻¹	+	20	15	+	+	+	
12. <i>I</i> in phosphate <i>M</i> : 3 · 10 ⁻²	+	20	15	— ¹	— ¹	— ¹	

¹ In these cases it was impossible to demonstrate any changes of course in the photosynthesis time curves in comparison with the time curve from plant material first dried and thereupon submerged in pure water (Figure 1, curve B).

² If the duration of the illumination is not 15 but 30 minutes no gush is registrable.

EDTA (ethylenediamine tetraacetic acid). Neither of the latter substances showed any effect whatsoever in the experiments here described (Table 1).

Cyanide is known to have an inhibiting effect on the zinc containing enzyme carbonic anhydrase. As cyanide is inducing a gush one might assume this enzyme to be the one involved in the transfer of CO₂ from acceptor I to photosynthesis proper. Carbonic anhydrase being specifically inhibited by sulphanilamide (Keilin and Mann 1940), experiments were made adding this substance to the plant material used; however, no effect whatsoever could be found (Table 1). In this connection it should be mentioned that phenylurethane has an inhibiting effect on carbonic anhydrase similar to that of cyanide (Meldrum and Roughton 1934), but the two substances affect the time curve of *Polytrichum* in different ways. The former, in contrast to the latter, appears not to affect the transfer of CO₂ from acceptor I (cf. Figure 2). Hence it must be concluded that carbonic anhydrase plays no part in the process of photosynthesis in general, nor in the induction phase in particular,

at any rate not in the case of *Polytrichum*. The experiments also show that the transfer of the carbon dioxide taken up by acceptor I to the photosynthetic process proper appears not to take place in the form of CO_2 via the carboxylation enzyme.

Concerning the identity of the acceptor I it is hardly possible to make any definite pronouncement at the present time. On the other hand, it ought to be mentioned that apart from ribulose diphosphate a number of biological occurring substances capable of taking up carbon dioxide are known. Among these are pyruvic acid and α -ketoglutaric acid, which are thus converted into malic acid and isocitic acid, respectively. Both reactions are reversible and require the presence of reduced coenzyme II (TPNH). Hence it is reasonable to assume that they would be strongly inhibited by iodoacetamide, which is certainly not the case with the uptake of carbon dioxide in acceptor I. The reactions mentioned have been described in detail by Vishniac, Horecker, and Ochoa (1957). It has further been demonstrated that phosphoenol pyruvic acid is able to take up CO_2 , forming oxalacetate and inorganic phosphate. This process is catalyzed by an enzyme containing active sulphydryl groups (Bandurski 1957). Hence it would, in this case as well, be reasonable to assume that iodoacetamide would have a strongly inhibiting effect (cf. also Kunitake, Stitt and Saltman 1959). Nitschporowitsch (1959) mentions in a review article that Kusin and Boitschenko have reported polyuronic acids to function as primary CO_2 acceptors, and that Nesgoworwa has found proteins able to function in this capacity. Finally, Metzner, Simon, Metzner and Calvin (1957) discovered in *Scenedesmus* an unstable CO_2 fixation product which appears to be different from the one formed from CO_2 and ribulose diphosphate (cf. Kasprzyk and Calvin 1959 who could not confirm this discovery). All things considered, plants contain a number of possible carbon dioxide acceptors, and the possibilities are not exhausted by the compounds mentioned above (cf. Gibbs 1959).

Thus we may record as a preliminary result of the present study that acceptor I takes up carbon dioxide immediately upon the onset of illumination. Under normal conditions, *i.e.*, when no form of inhibition affects the over-all process of photosynthesis, the total amount of CO_2 taken up by acceptor I is at $14\text{--}15^\circ$ transferred to the regular photosynthetic process within 5 to 6 minutes. If the illumination is interrupted before the full amount has been transferred, a larger or smaller fraction of the carbon dioxide taken up will be given off again in the form of a CO_2 gush. If the photosynthesis proper is inhibited and the transfer therefore blocked, the CO_2 retained by acceptor I will remain there throughout the light period, only to be relinquished in the form of a gush during the following dark period. If ordinary photosynthesis (acceptor II photosynthesis) is only partly

inhibited two possibilities exist. Either the inhibition is of a type which affects both the transfer and photosynthesis, in which case a CO_2 gush occurs immediately when the light source is removed; or it is of a type which, although it affects several of the individual reactions taking place during photosynthesis, has no effect on the transfer from acceptor I, in which case no CO_2 gush can be registered upon cessation of illumination. The first type is encountered when the inhibition of photosynthesis is caused by sufficiently high temperatures (45 to 50°), by narcotization at high concentrations of carbon dioxide (20%), or by treatment of the plants with cyanide, arsenate or iodoacetamide. The second type of inhibition is found when photosynthesis is inhibited by means of 2,4-dinitrophenol or phenylurethane. These substances are known among other effects to inhibit the oxidative phosphorylation and the reduction of oxidized cytochrome; hence it appears reasonable to assume that these processes are not involved in the transfer to acceptor II of the carbon dioxide taken up in acceptor I.

As cyanide and arsenate are known to have inhibiting effects on diphosphoribulose carboxylase this enzyme might have been involved in the process of transfer of carbon dioxide from acceptor I; but as, according to Weissbach *et al.*, the said enzyme is inhibited by phosphate as well, and this ion appears to be of no effect on the process of transfer, it may be assumed that this transfer consists in not only a yielding of the carbon dioxide to another acceptor (ribulose diphosphate), but that the carbon dioxide is carried in the form of an intermediate containing several carbon atoms.

Whereas the various chemicals with which the plants have been treated may be said to be more or less specific inhibitors for a number of enzymes, this is not true of the heat treatment used and of the use of a photosynthesis medium containing 20% of carbon dioxide. However, it may be said, and rightly so, that the lability of various enzymes to heat varies to a great extent, and hence it is quite possible that certain enzymes may be more strongly inhibited by heating to 45 to 50° for 3 minutes than are certain others. A 15° photosynthetic gas containing 20% of CO_2 has a marked narcotizing effect on the photosynthesis of leaves of potato and of beet, and this effect may be assumed to originate from adsorption phenomena on the phase boundaries of the grana of the chloroplasts (Gabrielsen and Vejlby 1959). Hence it might be considered possible to paralyze the transfer of CO_2 or of the corresponding intermediate from acceptor I through this type of adsorption. But by examining curve *U* (figure 7) in greater detail one observes that the CO_2 gush registered during the terminating dark period is relatively small compared to the 1 minute peak. If the illumination period is extended from 15 minutes, as in curve *U*, to 25 minutes, the CO_2 gush in the following dark period has disappeared. Hence it appears reasonable to assume that the

inhibition of the process of transfer caused by the high concentration of carbon dioxide is less pronounced than the inhibitions observed in the cases of heat treated plants and of those subjected to cyanide, arsenate, and iodoacetamide poisoning.

Summary

The photosynthesis time curve for the moss species *Polytrichum attenuatum* was drawn by means of the gas thermal conductivity method. It was shown that a CO₂ gush can be registered when the light is switched off after the completion of the induction phase, *i.e.*, following 15 minutes of illumination, in the following cases. 1) When the plants during the photosynthesis measurements are surrounded by air to which has been added 20 per cent of CO₂ or, when prior to the measurements, which then take place in atmospheric air with the addition of 3 per cent CO₂, they have been either 2) given heat treatment (3 minutes at 45 to 50°C) or 3) submerged in solutions of cyanide, arsenate or iodoacetamide. If they have been submerged in a similar way in solutions of 2,4-dinitrophenol or phenylurethane no gush appears. Treatment with phosphate, sulphanilamide, cysteine, and EDTA appears to be of no effect whatsoever.

It is concluded that the occurrence of the peak of the time curve is due to the uptake of carbon dioxide by an acceptor of unknown nature which we call acceptor I from which the CO₂ absorbed is transferred to the normal assimilation process in the form of an intermediate containing several carbon atoms.

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Transpiration and Water Movement in Young Wheat Plants

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1. Introduction

In studies concerning the transpiration from plants, in particular under ecological conditions, the so-called quick weighing method, consisting in determination of the weight loss from detached leaves or shoots over short periods of time, has found extensive use. Most of our present knowledge of transpiration ecology originates from studies of this kind. The method can be used only when the transpiration rate does not change during the measuring period due to the severing of leaves or shoots from their connections to water supplying organs. Iwanoff (1928) doubted the fulfilment of this requirement, being able to demonstrate an increase in transpiration from shoots, following immediately upon their removal from the plant. Subsequently the question of the suitability and reliability of the quick weighing method has been studied by several authors, with varying results. In most cases the transpiration was found not to have been changed by separating the leaves or the shoots from the rest of the plants. Stocker (1956) has reviewed these studies and their results. Stålfelt (1944) pointed out an important defect in the majority of the work concerning the applicability of the quick weighing method, *viz.*, it does not suffice to merely study the course of transpiration after the organ has been cut off, in order to ensure a proper evaluation of the results it is necessary also to know the rate of transpiration prior to cutting.

The difference in the results obtained from the methodological studies is probably due to the fact that physiologically very different species of plants

were used, or else plants which prior to the experiments had been subjected to dissimilar conditions of water supply; hence their transpiration and possibilities of transpiration had been extremely dissimilar. Water culture plants, which may be assumed to have uniform and reproducible possibilities of water uptake and consequently uniform hydration, appear not to have been used. Nor do studies appear to have been made concerning the importance of placing the cutting near to or far from the transpiring organ, which must cause differences in the capacity of accessible water reservoirs or in water conductivity resistance.

The development of the corona hygrometer which makes it possible to study transpiration phenomena in details by means of a gas stream method (Anderson, Hertz, and Rufelt 1954) has provided basis for a more critical methodological examination of problems presented by the quick weighing method. The corona hygrometer reacts extremely rapidly to changes in the humidity of the surrounding air. Hence it provides a unique possibility of studying changes in the transpiration during continuous measurements on one and the same plant material, before, during, and after interference with the pathways of the water supply inside the plants.

2. Methods

Experimental principles. A uniform plant material was used for the experiments, i.e., 7 to 10 days old wheat plants of the variety Skandia III, cultivated in water cultures. Before and during the experiments the plants were to the greatest possible extent treated identically in all respects. During the measurements proper the transpiration was raised to a high, stable level maintained by means of an ample water supply and initiated by illumination and a low relative humidity of the experimental gas. In contrast to the transpiration conditions, which were thus kept constant throughout the experiment, the water supply to the transpiring parts of the plants were affected by cuttings made under varying conditions and at different places in stems and roots. The cuttings were made either (A) in air or (B) under water, in one of three different places in the pathway of water transport, viz., 1) through the stem immediately above the seed, 2) through the root immediately below the seed and 3) through the root at a distance of about 3 cm below the seed. Thus the six resulting treatment combinations (A 1, 2, 3 and B 1, 2, 3) represent transpiring organs with different possibilities of water uptake after cutting.

Plant material. The wheat plants were grown in water culture according to the following plan. 5 g. of seeds (about 70 usable seedlings after 48 hours) were soaked in distilled water for 2 hours and subsequently sown in a large petri dish on several layers of filter paper moistened with distilled water. The petri dish containing the seeds was kept for 48 hours at a temperature of 20 to 21°C. Additional water was supplied at intervals, maintaining the level at just above the filter paper throughout the germination period.

The plants were now ready for water culture. Their roots were carefully put through holes in a plastic slab measuring $10 \times 10 \times 0.5$ cm. and having a total of 60 holes each of a diameter of 4.5 mm. The plastic slab acts as a lid covering a cubic glass jar containing about 500 ml of nutrient solution composed according to Olsen (1950). The culture vessels were placed in a green-house where in addition to daylight they could be supplied with electric light. Throughout the first 24 hours the plants were kept under a glass cover lined with moistened filter paper. The nutrient solution was renewed every other day.

The plants required for experiments were carefully loosened from the plastic slab (plants with damaged roots were rejected) and were placed in the plant chamber of the corona hygrometer by means of modelling wax which was also used to seal the lower opening of the chamber. The chamber was of the type used initially by Anderson, Hertz, and Rufelt (1954). At the beginning of the experiments the roots were placed in nutrient solution through which was bubbled CO_2 free atmospheric air. The weight of the plant material in the chamber (fresh weight of leaves) was from 0.40 to 0.80 g.

Transpiration measurements. The corona hygrometer used in the present study was modelled on the Swedish one described by Anderson, Hertz, and Rufelt (1954) and Anderson and Hertz (1955), with certain modifications concerning the air stream through the apparatus and the electronic parts of the apparatus.

Since it appeared to be desirable to use gas of a constant and known composition we chose compressed air from a cylinder. However, this type of gas is characterized by having a very low moisture content, and consequently a tube containing a saturated solution of magnesium acetate was placed in front of the sulphuric acid tubes in which the final humidity conditioning took place. Thus before reaching the place of final conditioning the gas stream attains a reasonable moisture content. The final humidity conditioning is done according to Anderson, Hertz, and Rufelt by means of sulphuric acid-water mixtures contained in 1.3 m. long glass tubes through which the air is passed over the mixtures; bubbling must not take place. In spite of the preliminary conditioning the sulphuric acid-water mixtures were found with time to change their composition; hence the true moisture content of the air was determined at suitable intervals by means of a dew point apparatus for streaming gases (make Casella), a method recommended by Gregory and Rourke (1957). Due to temperature fluctuations within the laboratory the conditioning tubes were placed inside a large water bath (250 litres) the temperature of which was regulated within an accuracy of $\pm 0.05^\circ$. Hence it was made possible to express the moisture content of the air in terms of the partial pressure of the water vapours.

Virgin (1956) recommended the insertion of an electrostatic filter between the plant chamber and the corona cell, in an attempt to protect the cell against dust particles. However, this type of electrostatic filter was found under certain unfavourable conditions to cause electric disturbances (through condensation and subsequent corrosion). Consequently the electrostatic filter was rejected in favour of a sintered glass filter, which was found to be extremely useful.

A constant voltage transformer supplied the current for the high voltage source for the corona and for the impulse counter which are both sensitive to variations in the mains voltage. In the impulse counter which was constructed according to the specifications of Anderson and Hertz a set of resistors was substituted for the potentiometer R_2 determining the time constant of the apparatus. The resistor R_3 which also affects the time constant was excluded due to the fact that the mV-recorder

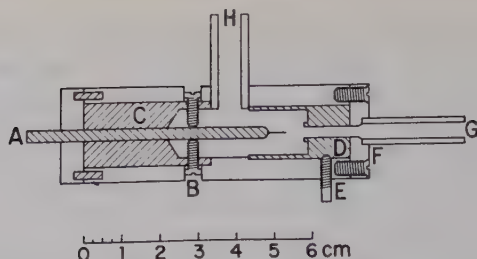


Figure 1. Section through new corona cell. All parts cross-hatched are made of brass, the remaining parts of acrylic plastic. The needle electrode A is kept in position by means of the 3 centering screws B in the electrode seating block C, which at the same time acts as a suitably air-tight closing of one end of the cell forcing the air stream to escape by way of the tube electrode D. This is being kept in position in the plastic housing by means of the screw E and of the end cover F which also keeps the air escape tube G. The air is introduced through the tube H. The high voltage leads are connected to the free end of the needle electrode and to the screw E.

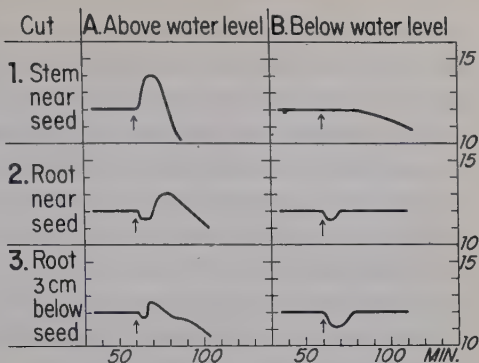
(the Speedomax) used contains a similar possibility for regulation. Upon the advice of Dr. Hertz the connections to the corona cell were made in the form of two independent leads placed as far as possible from each other because of their mutual capacity. The connections from the electrodes were shielded. The arrangement of the corona cell was also modified slightly as the initial threaded holes in the plastic material were found to wear out eventually. Hence the centering of the needle electrode became extremely difficult, and eroded plastic particles adhered to the electrodes and thus caused endless disturbances. The arrangement of the modified corona cell appears in Figure 1.

Transpiration conditions. All of the experiments were made at 20.0°C . The plants were illuminated by means of a photo lamp (Philips Argaphoto B) placed in an ordinary photographic aluminium reflector; the distance between the incandescent wire and the plant cell was 45 cm. A heat radiation filter consisting of a glass basin containing 11 cm. of water maintained at a temperature of 14° throughout the experiments was inserted between the lamp and the plant cell. The light intensity reaching the plants was about 12,000 lux. The gas passed into the plant chamber was CO_2 free atmospheric air, water vapour partial pressure 8.7 mm. Hg (relative humidity about 50 per cent at 20°). The rate of the air stream through the plant chamber past the leaves was maintained at a value of 1.25 to 1.33 m/min. During the experiments the rate was controlled by means of a rotameter (Rota, Aachen).

3. Results and Discussion

The curves in Figure 2 show the course of the transpiration when the water passage through the plant is disturbed by cutting under various conditions, and the water uptake in this way is either suspended altogether (cutting in air) or changed radically (cutting under water). The arrows under the curves indicate the time of interference. A reaction to the cutting can be

Figure 2. *Changes in the transpiration of wheat plants induced by disconnection of conducting tissue under different conditions. The axis of abscissas shows time in minutes from the onset of illumination. The ordinates represent the humidity of the air leaving the plant chamber, expressed by the partial pressure of the water vapour. The arrows indicate the time of cutting. Each curve is based on 3 experiments.*



observed only after an interval of more than a minute, *i.e.*, the period of time involved in the passage of the air from the plant cell to the corona cell.

If the cutting is made under water and through the stem (*B 1*) the transpiration remains constant for a period of about 20 minutes. A slow decrease then sets in, representing the fact that the plants can no longer take up sufficient water to maintain the initial rate of transpiration. Similar experiments carried out at a higher relative humidity of the air used in the experiment have shown that under these transpiration inhibiting conditions the plants are able to maintain their initial transpiration rate for several hours. If the roots are cut, also under water (*B 2* and *B 3*), transpiration shows a temporary decrease, subsequently adjusting itself to its initial rate which is then maintained long after the conclusion of the experimental period shown in the figure.

If the cutting is made in air (*A 1, 2, 3*) the rate of transpiration is found to increase to a greater or lesser extent regardless of which organ is affected. The increase occurs after a shorter or longer interval: if the stems are cut the increase sets in almost immediately, whereas if the roots are cut, transpiration is found to undergo a transient decrease matching that occurring in roots cut under water. In all three cases the increased transpiration is maintained only for a relatively short period of time corresponding to the water reservoirs available to the transpiring part of the plant, and eventually the rate of transpiration decreases gradually to values far below the initial one. The final decreases in transpiration are most abrupt in plants cut through the stems (*A 1*), and in most cases the plants lose water in such quantities that they are completely flaccid at the conclusion of the experiment. Plants, the roots of which have been cut 3 cm. below the seed (*A 3*), on the other hand show a considerably slower decrease in transpiration due to their somewhat more extensive water reservoirs. After the experiments the root-cut plants were fairly turgid.

Stålfelt (1944), like Iwanoff, followed the course of transpiration before and after cutting, and his failure to find an increase in transpiration in all his experiments must probably be attributed to the fact that the plants used had different water reserves. Stålfelt used pot-grown plants but prior to the experiments the pots and the soil were removed to reduce the weight, and the root was covered in water-tight wrappings. By this procedure it is hardly possible to avoid damaging the finer roots to a greater or lesser extent, and this may have caused differences in the experimental conditions.

In the present study we used plants grown in water culture, and hence it is reasonable to assume that the plants were well supplied with water, as were those of Iwanoff, which had been thoroughly watered prior to the experiments. An increase in transpiration was indeed found in all experiments involving cutting in air immediately or shortly after the process of cutting. Hence it appears to be reasonable to assume that in order to find an increase in transpiration upon cutting it is necessary to ensure that the transpiring parts of plants have ample available water reservoirs and hence the possibility of transpiring to a reasonably high extent. Experiments with the corona hygrometer have indeed shown that plants with reduced transpiration (due to high relative humidity) show no increase in transpiration upon cutting.

Usually the sudden increase of the transpiration is explained in terms of the cutting releasing the stress to which the water phase within the conductive tissues of the plants is subjected due to the resistance of the vascular elements to the transpiration flow. Hence water deficient plants might be expected to show more pronounced abrupt changes in their transpiration after cutting than do plants with a more ample internal water supply.

The translocation of water through the conducting tissues of the plants may be assumed to follow a modification of the Hagen-Poiseuilles law:

$$(1) \quad V = \frac{p N \pi r_a^4 t}{8 L \eta}$$

where V is the amount of water, p the reduction in pressure, N the number of capillaries in stem or root, r_a is the average radius of the capillaries, L the length of the pathway through the conducting tissue, t the time and η the viscosity (Hylmö 1958). The rule may be further converted into

$$(2) \quad p = \frac{V}{t N \pi r_a^2} \cdot \frac{8 L \eta}{r_a^2}$$

This formulation allows a direct comparison with Ohm's law, the reduction in pressure, p , being equal to the current (amount of water per unit of time and per cross section of the vascular elements) multiplied by the resistance.

Hence the over-all translocation of water through the plants may also be considered as a phenomenon obeying Ohm's law (van den Honert 1948), the driving forces — the potential — being transpiration and root pressure, while the resistance within the system consists of a series of components such as, *inter alia*, the resistance at interface (soil) water-root, that in the conducting tissues of the plant, and that at the interface leaf-atmosphere; among these the latter reach the highest resistance value by far. The ratio between reduction in pressure and resistance determines the current, in this case the transpiration. The cutting removes certain components of the resistance (L in equation 2 is reduced) and hence the resistance to water translocation within the plant decreases.

However, the change in resistance caused by the inactivation of part of the conducting tissues through cutting cannot directly cause the increase in transpiration. As mentioned above the resistance in the vascular elements is very slight compared to the resistance found at the interface between the cells of the mesophyll and the free atmosphere. This resistance might be assumed to be affected either 1) by a reduction of the resistance towards the passage of water through the cell walls of the mesophyll, *i.e.*, a change in permeability, or, 2) by a change in the resistance to diffusion, *viz.*, a change in the aperture of the stomata. Another possibility of course is a simultaneous functioning of the two processes.

Since the change of the aperture of the stomata must be assumed to be the process capable of causing the greatest reduction of the resistance, and hence is the one most likely to offer an explanation of the rapid and steep increase in transpiration observed, we shall here discuss this possibility in further detail.

The stomata of the wheat leaf are found on the upper as well as on the lower surface in rows in the epidermis situated between the almost parallel bundles of conducting cells. Thus the connecting pathways from the guard cells and the subsidiary cells (the stomatal apparatus) to the water carrying conducting elements are but short. In all probability the translocation of water takes place through the cell walls of the mesophyll and the epidermis cells (Strugger 1940).

As mentioned above, the cutting releases the stress in the conducting cells, and although this does not cause any considerable reduction of their resistance it may be assumed that the sudden change is transmitted through all of the water phase of the plant and also via the water in the cell walls to the guard cells and the subsidiary cells. Thus the value of the diffusion pressure deficit becomes lower in the area surrounding the stomatal apparatus which consequently is able to take up water, increase its turgor, and hence its aperture. An increase in the aperture causes a reduction of the diffusion resistance and

hence in increase in transpiration. In order for the reaction mechanism described here to function it is necessary that the diffusion of water into the guard cells can take place rapidly. The path of diffusion being very short, one appears to be justified in considering this requirement fulfilled. It should be possible to demonstrate this increase in the aperture of the stomata by direct observation; but we have not been able to do so yet, as we have not finished developing an appropriate method for microscopic measurements.

The theory proposed here presupposes the failure to produce an increase in transpiration when the stomata are fully open. A number of the data collected seem to indicate that this logical condition is fulfilled. Thus the transpiration in very humid air when the stomata are fully open at the light intensity used is not changed upon disjoining of the conducting tissues of the plant.

The increase in transpiration causes an increase of the water consumption which in the case of severing of the conducting elements in air can be maintained only for a short period of time determined by the capacity of the water reservoirs in the plant. Hence the increase in transpiration is necessarily followed by a decrease during which the stomata may possibly close due to water deficiency, corresponding to the "hydroactive closing" described by Stålfelt (1929, 1956).

The brief interval of reduced transpiration found in all experiments involving disconnection of the conducting tissues of the roots can probably be attributed to termination of the root pressure. The root pressure is a driving force in the translocation of water within the plants and codetermines the rate of water rise (g. per cross section and unit of time). A cut through the root will temporarily reduce the driving forces more than it does the resistance, and hence the rate of water rise is reduced, and so is the rate of transpiration. However, in the present case where the plants were young wheat seedlings the root pressure may be assumed to be of secondary importance only for the total translocation of water, the changes in transpiration induced by root cutting (Figure 2, A 2, 3; B 2, 3) being of brief duration only and being absent altogether if the stem and not the root is cut (A 1, B 1). Cutting through the root under water does not produce any permanent reduction of the transpiration (B 2, 3). As compared to the experiments in which the cutting was done in air the root pressure may be said under the conditions used here to be able to compensate only the resistance within the root proper, at the interface root-stem, and possibly in the very lowest part of the stem.

Rufelt (1956) studied the importance of the root pressure for transpiration by means of the corona hygrometer (cutting experiments, poisoning experiments and experiments in which the root pressure was inhibited by increasing the osmotic pressure of the nutrient solution). His results seem

to indicate a greater importance for transpiration of the root pressure than that discussed above. However, it must be emphasized that the conditions under which transpiration took place were different in the two studies, in that Rufelt used a light intensity of 65,000 lux (as compared to about 12,000 lux in the present study). Furthermore, it is seen from Rufelt's curves that the air leaving the plants was drier, its relative humidity only rarely exceeding 50 per cent, whereas in the present study it was above 85 per cent in all cases. Probably the combined effect of these conditions has been a water deficit in the leaves and stems of Rufelt's plants, and consequently they have been more dependent on the root pressure. Curve A in Rufelt's Figure 3 supports this assumption, as the variations in transpiration here must be explained by a deficit compensated by partial closing of the stomata.

A common feature of the results obtained by Rufelt and the present ones is that they leave no doubt as to the fact that a disconnection of the water conducting tissues of the plants may produce rapid and considerable changes in the rate of transpiration. The observation made by Iwanoff of a sudden increase of the transpiration in cut plant parts has been fully confirmed by means of a more perfect experimental technique. Hence the results appear to indicate that the quick weighing method is not applicable in all circumstances, and that it is hardly possible to control its results with sufficient accuracy by weighing even not when very short determination periods are introduced. Hence transpiration results obtained by the quick weighing method should be considered only with a certain scepticism.

Summary

The corona hygrometer has been used to study the changes in transpiration induced by disconnecting the conducting tissues in 7 to 10 days old water culture plants (wheat). The disconnections were performed either in air or under water by cutting in any one of the following places 1) through the stem immediately above the seed, 2) through the root immediately below the seed or 3) through the root at a distance of about 3 cm from the seed. The increase in transpiration found to result from all cuttings performed in air is attributed to the sudden reduction of the resistance which induces a stomatal reaction in the leaves causing a decrease in the diffusion resistance from the interior of the leaf to the atmosphere. The transient transpiration decreases resulting from root cuttings are attributed to the sudden inactivation of the root pressure. When the cutting is done in air a decrease in transpiration occurs towards the end of the experiment due to desiccation and possibly accompanied by "hydroactive closure" of the stomata.

In general the experiments show that the disjoining of conducting elements may cause extensive changes in the transpiration. In order for the changes to take place it appears to be necessary that the plants are well supplied with water and that they have a suitable initial rate of transpiration.

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Uptake of Phosphate and Sulphate by Wheat Roots at Low Temperature

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Introduction

The uptake of salts by plant roots and by discs of storage tissue can be resolved into two main processes, namely a relatively rapid entry into what has been termed the apparent free space (A.F.S.) and a slower accumulation into the vacuoles, (see reviews by Epstein, 8, Briggs and Robertson, 1, Sutcliffe, 18, and Laties, 13). Whereas the latter process is usually thought to occur by combination of the ions with endogenously produced carrier substances, the passage into the free space is believed to be dependent on physical factors such as diffusion and exchange. Exchange-adsorption phenomena within the free space are relatively important for cations, because of the presence of large numbers of non-diffusible anions in the free space, (Briggs and Robertson, 1, Briggs, Hope and Pitman, 2). On the other hand, anion exchange sites are present at much lower concentrations (Epstein, 7, Briggs *et al.*, 2, Laties, 13, 14). It is possible, therefore, that these sites may be those directly concerned with active transport.

The object of the experiments reported in this paper was to determine the relative importance of exchange-adsorption phenomena in wheat roots for phosphate and sulphate. The uptake of P^{32} -phosphate and S^{35} -sulphate by excised wheat roots at 1° was studied over a range of concentrations.

Experimental Methods

The experimental material was wheat, variety "Cross 7". Techniques which have been described earlier (Butler 4) were used for seed germination and growth of the seedlings in nutrient solution in a controlled environment cabinet. The nutrient solution had the following composition:

1 mM- KH_2PO_4 , 1 mM- $\text{Ca}(\text{NO}_3)_2$, 0.5 mM- MgSO_4 , 20 μM - MnSO_4 , 1 μM - $\text{B}(\text{OH})_3$. Iron versenate, prepared by the method of Jacobsen (10) was added at a concentration of 10 μM .

Plants were used for the absorption experiments at an age of 7—14 days. They were slowly chilled to 1° overnight; rapid chilling caused irreversible changes in the roots, particularly plasmolysis of root hairs and coagulation of cytoplasm.

The uptake of anions was then studied by placing excised roots (up to 10 cm. long) in test solutions containing radioactive material. The ratio of test solution volume to root fresh weight was always 100 or greater, so that insignificant changes in concentration of the solution occurred during the absorption period. All solutions were aerated, this also ensuring rapid mixing.

P^{32} was obtained as carrier-free orthophosphate and S^{35} as carrier-free sulphate from the Radiochemical Centre, Amersham, England; they were added to the test solution in amounts of 0.5—1.0 mC. per l. In each experiment, the total amount of P^{32} or S^{35} was constant, and independent of the total concentration of carrier phosphate or sulphate.

After the desired equilibration time had elapsed, the roots were freed from surface moisture by pressing firmly between sheets of blotting paper. The root fresh weight was rapidly determined and the roots ashed by the method described earlier (Butler 5). In the case of P^{32} , radioactivity was measured by a liquid-counting technique (Butler 5); for S^{35} , aliquots were precipitated as BaSO_4 , plated out and counted at infinite thickness, according to the method of Kylin (11).

Results

Uptake of labelled phosphate at different concentrations of carrier phosphate

This was determined using roots of plants which had been maintained at a normal level of phosphate during growth. After chilling the plants to 1° overnight, the excised roots were placed for 2 hr. at 1° in KH_2PO_4 solutions ranging in concentration from 10^{-5} M to 10^{-2} M, adjusted to pH 5.6 with KOH. The roots were then transferred to two sets of P^{32} -labelled KH_2PO_4 solutions of the same concentrations and pH for the times shown in Table 1. One set of labelled solutions contained 5×10^{-5} M dinitrophenol, (DNP), which inhibits active salt uptake; at this concentration and pH the inhibition is almost completely reversible for wheat roots (Butler 6).

Table 1. *Uptake of labelled phosphate from different concentrations of phosphate into excised roots at 1°*. Test solutions were 10^{-2} – 10^{-5} M KH_2PO_4 , adjusted to pH 5.6, having 1 mC per l. P^{32} . One series of solutions contained 5×10^{-5} M DNP.

Concentration (M)	% Activity per g. root (relative to 1 ml. test solution)		
	Untreated		DNP-treated
	60 min.	90 min.	112 min.
2×10^{-5}	128	156	127
3.5×10^{-5}	74	99	72
1×10^{-4}	72	83	67
3.3×10^{-4}	28	29	31
1×10^{-3}	23	22	21
3.3×10^{-3}	25	—	23
1×10^{-2}	21	19	19

In Table 1 are given the levels of labelled phosphate found in the roots after exposure to the test solutions. The results here and in subsequent experiments are expressed as

$$\frac{\text{activity/gm. root}}{\text{activity/ml. solution}} \times 100$$

Uptake of labelled phosphate with time with different levels of carrier phosphate

Two experiments were carried out with roots of plants which had been maintained at normal phosphate status during growth.

(a) The plants were chilled to 1° overnight in the normal nutrient solution and the excised roots were transferred directly to solutions at 1° containing a series of concentrations of labelled KH_2PO_4 , adjusted to pH 5.6 with KOH. The results are shown in Figure 1 A.

(b) The plants were chilled to 1° overnight in nutrient solutions containing a series of concentrations of KH_2PO_4 , adjusted to pH 5.6 with KOH, with other nutrients at the usual concentrations. The excised roots were then placed in labelled solutions of the same composition and concentration at 1°. To a duplicate solution containing 10^{-3} M phosphate was added 5×10^{-4} M DNP two hours before the roots were transferred to the labelled solutions. The results are shown in Figure 1 B.

Uptake of labelled phosphate with time; roots grown in phosphate-deficient solutions

Two experiments were made with roots which had been grown in nutrient solutions deficient in phosphate (KH_2PO_4 was replaced by KNO_3). The roots were long and slender with subnormal roothair development and charac-

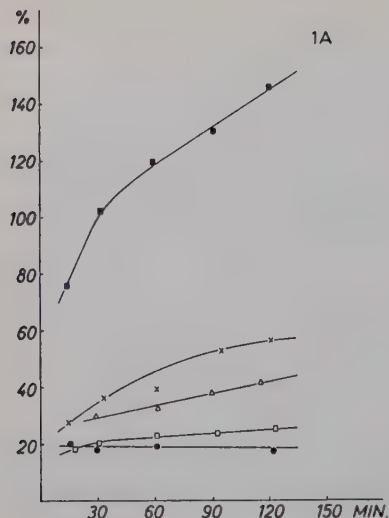


Figure 1 A.

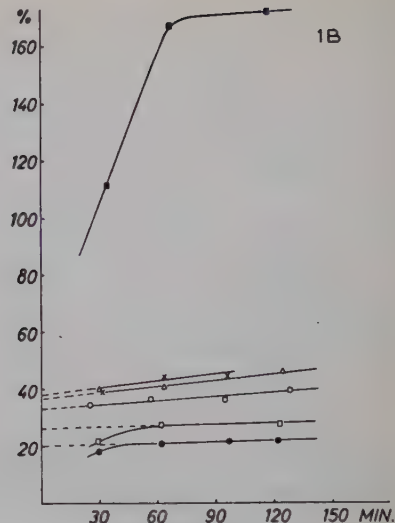


Figure 1 B.

Figures 1 A and 1 B. Uptake at 1° of labelled phosphate at different concentrations of phosphate by excised roots grown at normal phosphate status. On the ordinate activity % per g. root, relative to the test solution.

1 A. Chilled overnight in normal nutrient solution; uptake from solutions containing only phosphate.

1 B. Chilled overnight in nutrient solution with different phosphate levels; uptake from similar labelled solutions.

■ 10^{-4} M phosphate
 △ 10^{-3} M phosphate
 □ 6×10^{-3} M phosphate

× 6×10^{-4} M phosphate
 ○ 10^{-3} M phosphate with 5×10^{-5} M DNP
 ● 2×10^{-2} M phosphate

teristically-pointed root-caps. In both experiments the plants were chilled to 1° overnight in phosphate-deficient nutrient solutions and then transferred to the test solutions. In one experiment (Figure 2) the test solutions were 0.5 mM and 10 mM KH_2PO_4 , adjusted to pH 5.6; in the other experiment the same concentrations of KH_2PO_4 were used in the presence of normal concentrations of other nutrients. Similar results were obtained from both experiments.

Loss of labelled phosphate from roots

The loss at 1° of labelled phosphate taken up by wheat roots was examined briefly. Roots which had been in labelled 2×10^{-5} M KH_2PO_4 for 70 and 110 min. retained 59 and 65 per cent respectively of their radioactivity after 45 min. in distilled water. With roots which had been labelled 10^{-2} M KH_2PO_4 for 110 min., 18 per cent of the radioactivity was retained after 40 min. in

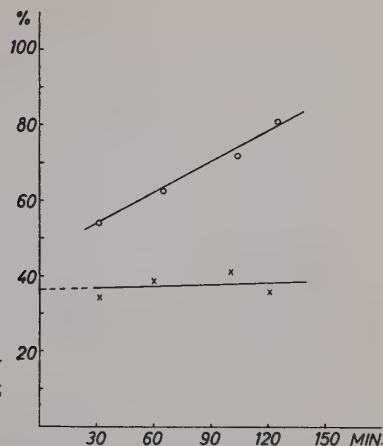


Figure 2. Uptake of labelled phosphate by excised phosphate-deficient roots at 1° . Circles, $5 \times 10^{-4} M$ phosphate; crosses, $10^{-2} M$ phosphate.

distilled water. Roots which had been in labelled $10^{-3} M$ KH_2PO_4 for 90 min. retained 8 per cent of their radioactivity after 60 min. in unlabelled KH_2PO_4 .

Uptake of labelled sulphate at different levels of carrier sulphate

Two experiments were carried out in which excised roots of normal sulphate status were equilibrated with a range of S^{35} -labelled K_2SO_4 solutions at 1° . As in the experiments with labelled phosphate, the plants were slowly chilled overnight; the excised roots were placed for 2 hr. in unlabelled K_2SO_4 solutions and then equilibrated in labelled solutions of the same concentration. Some of the roots were transferred to water for 60 min. after equilibration with labelled sulphate for 80 min. The results of one of the experiments are given in Table 1.

Discussion

In considering these experiments, it should be remembered that unit volumes of the test solutions always contained the same amount of radioactivity, irrespective of the total sulphate or phosphate concentrations.

In accordance with the evidence collected by Levitt (15) and Laties (13), the "apparent free space" is taken to comprise the cell wall volume and the film of liquid adhering to the root surface. Particularly for the higher solute concentrations, the first rapid phase of uptake should correspond to the "apparent free space". It will be seen from Table 1, Figure 1 B and Table 2 that for phosphate concentrations of $10^{-2} M$ and $2 \times 10^{-2} M$ and for most sulphate concentrations the "apparent free space" of normal roots was 18—20

Table 2. *Uptake of labelled sulphate from different concentrations of sulphate into excised roots at 1°, and subsequent loss to water.*

Concentration (<i>M</i>)	% Activity per g. root (relative to 1 ml. test solution)			A.F.S. %
	Sulphate 95 min.	Sulphate 120 min.	Sulphate 80 min. Water 60 min.	
2×10^{-5}	28	30	7.2	22
3.5×10^{-5}	21	19	7.5	—
1×10^{-4}	22	20	1.7	19
3.3×10^{-4}	23	21	1.5	21
1×10^{-3}	20	19	1.8	18
3.3×10^{-3}	18	19	0.6	18
1×10^{-2}	20	18	0.6	18

per cent. With phosphate-deficient roots (Figure 2), which are long, unbranched and slender with characteristic anatomy, values of 36 per cent were obtained for the "apparent free space" to 10^{-2} *M* KH_2PO_4 in two experiments.

At lower solute concentrations, there is a marked difference in the results for phosphate and sulphate. Whereas the amounts of S^{35} -sulphate taken up are nearly independent of sulphate concentration, considerably greater amounts of P^{32} -phosphate were taken up with decreasing phosphate concentration. If adsorption sites for a particular ion were present within the roots, they should contribute proportionately more to the uptake of labelled ion with decreasing concentration in these experiments. It can therefore be concluded from Table 2 that adsorption sites for labile binding of sulphate were present at a very low level (not greater than 10^{-6} *M*), in agreement with the results of Kylin and Hylmö (12) for wheat roots. Epstein (7) found a somewhat larger degree of labile binding of sulphate in barley roots.

For phosphate, the interpretation of the results is less straightforward. It will be noted that: —

(a) There was a relatively rapid uptake of P^{32} -phosphate in excess of that to be expected for movement into the "free space" during the initial 30 minutes, this amount increasing with decreasing concentration (Figures 1 A, 1 B and 2).

(b) A slower uptake of P^{32} continued over the remaining period, being more pronounced at lower concentrations.

(c) DNP reduced the rapid phase of P^{32} -uptake (Figure 1 B) and also the slower phase (Butler 6).

(d) The increased P^{32} -phosphate uptake at lower phosphate concentrations was observed both in the presence and absence of other nutrient salts at normal nutrient solution concentrations (Figures 1 A, 1 B).

(e) Appreciable quantities of P^{32} were retained by the roots after transferring to water or unlabelled phosphate.

The mechanisms which might play a part in the increased P^{32} uptake are metabolic accumulation, immobilisation by complexing with iron within the roots, esterification (to nucleotides or hexose phosphates) and adsorption exchange. Metabolic accumulation continues at a significant rate at 1° (Broyer 3, Laties 14) and, since the specific activity of the phosphate increased 1,000-fold in reducing the total concentration from 10^{-2} *M* to 10^{-5} *M*, accumulation would be more sensitively detected at the lower concentrations. On the other hand, because the plants were held at 1° for 16 hr. and (except in Figures 1 A, 2) also pre-treated with unlabelled solutions of the same composition as the labelled solutions, no "absorption shoulder" would be observed, (Laties 13, 14). Hence metabolic accumulation did not account for the increased rapid uptake at lower concentrations, but contributed to the second phase.

It is possible that some phosphate was immobilised within the roots by iron absorbed simultaneously (Figure 1 B) or immediately prior to the experiment. Foster and Russell (9) found evidence for such a phenomenon in barley roots during 24 hr. absorption experiments. Since iron was supplied in relatively low concentrations in chelated form in the present experiments, it is not considered likely that this effect was operative to any large extent.

The relative contributions of adsorption-exchange and esterification cannot be assessed from these experiments. The amount of esterification which occurred was not measured and the data presented on the loss to distilled water and to unlabelled phosphate of P^{32} taken up by the roots is limited. The loss to distilled water of 41 per cent of the P^{32} taken up from 2×10^{-5} *M* phosphate represented a loss of 70 units of radioactivity, 3.5 times greater than that corresponding to a free space of 20 per cent.

With regard to esterification, Loughman and Russell (16) showed that, of the P^{32} taken up from barley roots of low phosphate status from 3×10^{-5} *M* phosphate under glass-house conditions, 28 per cent was incorporated into organic fractions after 10 min. and 56 per cent after 60 min. Incorporation into nucleotides was more rapid than incorporation into hexose phosphates. The bearing which these findings have on the present experiments is uncertain, because the rate of esterification at 1° would be slower and because most experiments were carried out with roots of high phosphate status. Loughman and Russell (16) also found that DNP at strengths of 10^{-4} *M* and 5×10^{-5} *M* reduced incorporation into nucleotides of barley roots by one half, but did not affect incorporation into hexose phosphates. The reduction in the rapid phase of P^{32} uptake in the presence of DNP shown in Fig. 1 B might be due to such an effect.

Because the phosphate concentration of the soil solution may be less than 3×10^{-7} *M* and seldom exceeds 10^{-4} *M* (Russell 17), further work is desirable

on the relative contributions of adsorption exchange and esterification to phosphate uptake from very dilute solutions.

The author wishes to acknowledge the careful technical assistance of the late Miss C. L. Bray in this work.

Summary

The uptake of P^{32} -phosphate and S^{35} -sulphate by excised wheat roots at 1° has been examined over a range of concentrations from $2 \times 10^{-5} M$ to $2 \times 10^{-2} M$ for periods up to 130 minutes.

(1) The results at higher concentrations of both sulphate and phosphate were consistent with an "apparent free space" of 18—20 per cent for normal roots. For phosphate-deficient roots, the "apparent free space" was 36 per cent.

(2) Whereas the uptake of labelled sulphate was nearly independent of carrier sulphate concentration over the entire range, the uptake of labelled phosphate was markedly greater with reduction in carrier concentration. $5 \times 10^{-4} M$ dinitrophenol reduced but did not abolish this effect, which was concluded to arise from a complex of mechanism. Further work is required to establish the relative contributions of adsorption exchange and esterification, in particular.

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Utilization of Inulin and Starch by Three Soft Rot Bacteria

By

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Introduction

A number of microorganisms are known to attack starch (Buchanan and Fulmer 1928), hydrolysing it through dextrins to maltose and glucose, the glucose then being oxidatively or fermentatively dissimilated to other products. On the other hand, Tarr (1934) reported that the endospores of certain bacteria hydrolyse starch to maltose only. Similar results were reached by Doudoroff (1940) in his studies on the hydrolysis of starch by the extracellular enzyme of *Pseudomonas saccharophila*. Gray (1939) found that, under certain conditions, *Vibrio amylocalla* hydrolysed starch and dextrins to glucose only.

Starch is hydrolysed by a group of enzymes known as amylases or diastases. The amylase of wheat was probably the first enzyme to be discovered. In recent years a number of amylases have been obtained in crystalline form by Balls *et alii* (1948) and by Meyer *et alii* (1947, 1948). A great many bacteria show diastase activity (Johnston and Wynne 1935, Kerr 1943, Wilson *et alii* 1943).

Inulin is also attacked by several microorganisms (Tarr 1934, Finkle 1936, Doudoroff 1940). It has even been reported (Guillemot and Leroux 1938, 1939) that certain yeasts which do not ferment fructose slowly attack inulin.

The enzyme inulase hydrolyses the polysaccharide inulin to fructose. Pringsheim and Aronowsky (1940) prepared inulase from *Penicillium glaucum* cultured on a medium that contained inulin. Pringsheim and Hensel (1940) obtained it from *Aspergillus niger*. Several bacteria ferment inulin, but the enzyme system has not been systematically studied. Weidenhagen (1940) maintains that inulase is a β -heretofructosidase.

The experiments reported in the present work were thus planned with a view to throwing some light on the nature of the enzymes concerned with the utilization of starch and inulin by three closely related soft rot bacteria.

Material and Methods

The organisms used in this investigation were as follows:

1. *Bacterium aroideae*, (Townsend) Stapp; Dowson's strain, 66.
2. *Bacterium carotovorum*, (Jones) Lehmann and Neumann.
3. *Bacterium phytophthorum*, (Appel) Burgwitz.

The three cultures were kindly supplied by the Plant Bacteriological Laboratory of the Imperial College of Science and Technology, London.

In this series of experiments a basal medium of the following composition was prepared:

Asparagin	10	g.
MgSO ₄ · 7H ₂ O	2.5	g.
KH ₂ PO ₄	5	g.
Distilled water to	500	ml.

The various carbohydrates or mixtures of carbohydrates tested were dissolved separately in sterile distilled water at double the required concentration. Equal volumes of the carbohydrate solution and the basal medium were mixed. The full culture media so prepared were distributed into a series of sterile 150 ml. conical flasks at the rate of 10 ml. per flask and inoculated at the rate of 0.2 ml. of a suspension of each of the three bacteria being tested in sterile distilled water. The flasks were then incubated at 25°C for the specified time intervals at the end of which the carbohydrate contents of the various media were determined. The procedure used for the estimation of carbohydrates was that standardised by Maskel and his coworkers at the Botany School of Cambridge (cf. Said 1937, 1941, 1945).

Experimental Results

Utilization of starch by the three bacteria

In the first experiment of this series 2 % glucose or 2 % starch were added to the basal medium as carbon sources. Each of the three bacteria was successively sub-cultured daily on each of the above two media for a period of 7 days. At the end of each 24 hour interval the crop obtained from the growth of each bacterium was centrifuged, washed, and inoculated into fresh medium of the same basal composition with starch as carbon source. Each set of culture flasks was incubated at 25°C for 24 hours at the end of which the media were analysed for their direct and total reducing values.

The results of the analyses of the various media showed that starch undergoes rapid hydrolysis by the three bacterial organisms; the rate of uptake of the hydrolysis products is slower than the rate of hydrolysis, thus reducing

Table 1. Amounts of starch utilized from Culture Media in 24 hours by *B. aroideae*, *B. carotovorum* or *B. phytophthorum* initially subcultured on glucose or starch media and transferred daily to fresh media containing starch over a period of 7 days. Calculated as mgm. glucose per ml. medium.

Consecutive 24 hour Intervals	<i>B. aroideae</i>		<i>B. carotovorum</i>		<i>B. phytophthorum</i>	
	Glucose	Starch	Glucose	Starch	Glucose	Starch
1 <i>st</i>	7.66	7.40	6.38	6.64	5.49	5.10
2 <i>nd</i>	7.27	7.27	6.38	6.38	5.36	5.36
3 <i>rd</i>	7.40	7.40	6.13	6.64	5.10	5.49
4 <i>th</i>	7.02	7.66	6.13	6.38	5.36	5.36
5 <i>th</i>	7.40	7.66	6.00	6.13	5.49	5.10
6 <i>th</i>	7.02	7.27	6.13	6.38	5.75	5.36
7 <i>th</i>	7.27	7.40	6.38	6.64	5.36	5.49

sugars accumulate in the various media throughout the whole experimental period.

Continued subculture of the three bacteria in presence or absence of starch did not exert any substantial effect on the amounts of reducing sugars recovered in the various culture media of each bacterium.

The amounts of starch utilized from the various culture media during the consecutive 24 hour intervals by each of the three bacterial organisms when initially subcultured on glucose — or starch — containing media are calculated and presented in Table 1.

Table 1 shows that for each bacterium the amount of starch absorbed in 24 hours is more or less constant whether the organism is initially subcultured in presence or absence of starch, a result which would indicate that the enzyme concerned with starch utilization by the three bacteria is a constitutive one the stability of which is not affected to any appreciable extent by the presence of the substrate. In fact, the similar values for starch utilization by inocula obtained from crops grown in presence and absence of starch may be due to the stability of the enzyme concerned in the latter case on account of the presence of glucose, a hydrolysis product of starch, in the culture medium used for initial subculture. The results in Table 1 also show that *B. aroideae* utilized the highest amounts of starch from its media, *B. phytophthorum* the lowest with *B. carotovorum* occupying an intermediate position.

To test whether or not the enzyme concerned with starch hydrolysis is excreted into the culture medium, the three bacteria were inoculated separately into the starch containing medium, incubated at 25°C for 24 hours and the various media then analysed for their contents of reducing sugars. The culture media were then freed from their bacterial contents by centrifugation and reincubated for another 24 hours after which the various media

Table 2. Amounts of starch that disappeared from culture media inoculated with *B. aroideae*, *B. carotovorum*, or *B. phytophthorum* as affected by vitamin C. Calculated as mgm. glucose/ml. medium.

Organism	Starch	
	Without vitamin C	With vitamin C
<i>B. aroideae</i>	11.36	10.16
<i>B. carotovorum</i>	9.52	9.16
<i>B. phytophthorum</i> ...	7.70	8.09

were again analysed for their direct reducing values. The results of this experiment revealed the complete absence of any extracellular amylase in case of the three bacteria.

The accumulation of reducing sugars in the culture medium suggests that the enzyme concerned with starch hydrolysis is, in all probabilities, an amylase. The possibility of the mediation of a specific phosphorylase which is of wide distribution in animals, plants and microorganisms (Goddard and Meuse 1950, Hehre 1951, Peat 1951) is refuted on account of the fact that such enzyme attacks starch forming glucose-1-phosphate and leaving the residual part of the molecule in the culture medium. The fact that reducing sugars accumulated in the medium through bacterial growth indicates a hydrolytic process rather than phosphorolysis.

Ascorbic acid was found by Purr (1934) to inhibit the β -amylases of plants but does not affect their α -amylases. To test for the action of ascorbic acid on the enzyme concerned with starch hydrolysis in the bacteria under test, starch was added to the basal medium as a source of carbon. To a portion of the full medium so prepared ascorbic acid was added at a concentration of 100 p.p.m., the other portion was used as a control. The sterile culture media were then inoculated with bacterial suspensions at the rate of 0.2 ml. per 10 ml. medium. The various cultures were incubated at 25°C for 24 hours. The direct and total reducing values of the media were estimated before and after incubation.

From the results of the analyses of the various culture media the amounts of starch which disappeared from the various media being utilized by the bacteria or recovered as reducing sugars in the medium are presented in Table 2. The results show that vitamin C did not affect the rate of disappearance of starch from the culture media to any appreciable extent. This would indicate that the utilization of starch by the three bacteria is most probably mediated by an α -amylase enzyme.

Utilization of inulin by the three bacteria

In this set of experiments 2 % glucose or inulin were added to the basal medium. Each of the three bacteria was subcultured daily on the above two media for 7 successive days. Each bacterium was transferred daily to a fresh medium containing inulin as carbon source according to the method described earlier. Culture flasks were incubated at 25°C for 24 hours at the end of which the media were analysed for their direct and total reducing values.

The results of the analyses of the various media revealed that out of the three bacteria used in this investigation only *B. phytophthorum* induced rapid hydrolysis of inulin in its culture media. The rate of uptake of the hydrolysis products of inulin by *B. phytophthorum* is apparently slower than the rate of hydrolysis, thus reducing sugars accumulated in its culture media.

The amounts of inulin utilized by each of the three bacterial organisms from the various culture media in the consecutive 24 hour intervals are calculated and recorded in Table 3. The result which stands out clearly is the great amounts of inulin utilized by *B. phytophthorum* as compared to those utilized by *B. aroideae* or *B. carotovorum*. It is also to be observed that continued subculture of the 3 organisms in presence or absence of inulin did not materially alter the rate of utilization of this polysaccharide by the three bacteria, a fact which indicates that the enzyme concerned is a constitutive one which is not affected, to any appreciable extent, with regards its stability, by the presence of the substrate.

Hydrolysis of inulin may be mediated by β -heterofructosidase which attacks fructoside links or, alternatively, by a specific inulase enzyme. The fact that *B. aroideae* and *B. carotovorum* revealed β -heterofructosidase activity (Tolba and Ghanem 1954, 1959) may suggest that inulin is attacked by these two bacteria through the mediation of the β -heterofructosidase enzyme which is known to hydrolyze inulin some 5000 times slower than sucrose and hence

Table 3. Utilisation of Inulin in 24 hours from culture media of *B. aroideae*, *B. carotovorum* or *B. phytophthorum* when initially subcultured on glucose or inulin media and transferred daily to fresh media containing inulin over a period of 7 days. Calculated as mgm. glucose per ml. medium.

Consecutive 24 hour Intervals	<i>B. aroideae</i>		<i>B. carotovorum</i>		<i>B. phytophthorum</i>	
	Glucose	Inulin	Glucose	Inulin	Glucose	Inulin
1 st	0.63	0.63	0.40	0.40	4.16	3.83
2 nd	0.63	0.89	0.40	0.65	4.16	4.16
3 rd	0.63	0.89	0.27	0.40	3.83	4.16
4 th	0.63	0.63	0.40	0.40	3.83	3.58
5 th	0.89	0.40	0.27	0.27	4.16	4.21
6 th	0.63	0.40	0.27	0.40	4.16	3.83
7 th	0.63	0.89	0.27	0.40	4.16	4.06

Table 4. *D.R.V. of sucrose and inulin media inoculated with cells of B. phytophthorum heated to 60°C for 10 minutes and incubated for one hour. Calculated as mgm. glucose per ml. medium.*

Carbohydrate in the culture medium	D.R.V. of the medium		Increase in D.R.V. of the medium
	Initial	Final	
Sucrose	0.29	0.59	0.39
Inulin	0.00	0.88	0.88

the very small amounts of inulin utilized by each of the two organisms in 24 hours.

The direct reducing values of inulin and sucrose media inoculated with cells of *B. phytophthorum* heated at 60°C, after one hour incubation are given in Table 4.

The values recorded in Table 4, though rather small on account of the short duration of the experiment, show that inulin is hydrolyzed by *B. phytophthorum* at a somewhat faster rate than sucrose, a fact which strongly supports the hypothesis that *B. phytophthorum* hydrolyses inulin primarily by a specific inulase.

In a way of verifying the above hypothesis; *viz.* that inulin utilization by *B. aroideae* and *B. carotovorum* takes place through β -heterofructosidase while *B. phytophthorum* attacks it through the possible mediation of a specific inulase enzyme, the effect of fructose (known to inhibit β -heterofructosidase) on the utilisation of inulin by the three bacteria was studied in the following way:

2 % inulin or a mixture of 2 % inulin and 5 % fructose were added to the basal medium.

The two media were inoculated with each of the three bacteria, incubated at 25°C for 24 hours at the end of which the media were analysed for their direct and total reducing values. From the results of the analyses the amounts of inulin which disappeared from the various culture media are set out in Table 5.

Table 5. *Utilization of inulin from culture media of B. aroideae, B. carotovorum, or B. phytophthorum as affected by the presence or absence of fructose. Calculated as mgm. glucose/ml. medium.*

Organism	Carbohydrates in culture medium	
	2 % Inulin	2 % Inulin + 5 % Fructose
<i>B. aroideae</i>	0.67	0.00
<i>B. carotovorum</i>	0.60	0.00
<i>B. phytophthorum</i>	6.59	4.33

Table 5 reveals a complete stoppage of the utilization of inulin by *B. aroidae*, and *B. carotovorum* and a reduction in the rate of utilization of this polysaccharide by *B. phytophthorum* in presence of fructose. The complete stoppage in case of the former organisms is probably due to two factors, a) the availability of ready fructose and b) the inhibiting action of fructose on the β -heterofructosidase enzyme. The utilization of appreciable amounts of inulin by *B. phytophthorum* in presence of fructose indicates that inulin utilization by this organism is, at least partly, mediated by a specific inulase enzyme. The reduction in the amount of inulin utilized may be due to the availability of fructose.

To test for the possible production of an extracellular inulase enzyme of low activity by *B. aroidae* and *B. carotovorum*, the two organisms were inoculated separately into inulin medium and incubated for 24 hours at 25°C, the media were then analysed for their direct reducing values, freed from their bacterial contents, reincubated for another 24 hours and again analysed for their contents of reducing sugars.

The results of the above experiment indicated no hydrolytic effect of bacteria-free media on inulin, thus indicating that no extracellular enzyme is excreted by either of these 2 organisms.

B. phytophthorum culture media freed from their bacterial content also failed to reveal any hydrolytic power on inulin, thus indicating the absence of any enzyme concerned with inulin hydrolysis by this organism in the external medium.

Discussion

Utilization of starch

The results of the present investigation indicate that starch undergoes rapid hydrolysis in the culture media of the three bacteria being tested. The amounts of starch utilized by each bacterium in 24 hours are, more or less, constant whether the organism is initially subcultured in presence or absence of starch. This indicates that the enzyme concerned with starch utilization by the three bacteria is a constitutive one the stability of which is not affected to any appreciable extent by the presence of the substrate. In fact the similar values for starch utilization by inocula obtained from crops grown in presence and absence of starch may be due to the stability of the enzyme concerned, in the latter case on account of the presence of glucose, a hydrolysis product of starch, in the culture medium sued for initial subculture.

The rate of hydrolysis of starch by the three bacteria is not materially affected by the presence of vitamin C, known to inhibit plant β -amylase. This result would indicate that the enzyme concerned with starch hydrolysis is most probably an α -amylase.

Tests for the presence of an extracellular amylase enzyme proved negative in case of the three bacteria being tested. The large size of the starch molecule refutes the possibility of the uptake of starch unhydrolyzed.

The above results therefore suggest that starch is hydrolyzed by an amylase enzyme, most probably an α -amylase, located at the protoplasmic surface of the bacterial cells.

Utilization of inulin

Out of the three bacteria used in this investigation, only *B. phytophthorum* induced rapid hydrolysis of inulin in its culture media. The rate of uptake of the hydrolysis products of inulin by this organism is apparently slower than the rate of hydrolysis, and hence the accumulation of reducing sugars in the culture media. *B. aroideae*, and *B. carotovorum*, on the other hand, utilized very small amounts of inulin, presumably after being hydrolyzed on account of the inconceivability of the possible uptake of such large molecule without hydrolysis. Continued sub-culture of all three bacteria in presence or absence of inulin did not induce any effect on the rate of hydrolysis of this polysaccharide, thus indicating that the enzyme concerned in each case is a constitutive one. The small amounts of inulin utilized by *B. aroideae*, and *B. carotovorum* would indicate that the pathway of metabolism of this polysaccharide is through the action of β -h-fructosidase, the presence of which was established in these two organisms (Tolba and Ghanem 1954, 1959) and which is known to hydrolyze inulin some 5000 times slower than sucrose. Tests for extracellular enzymes for inulin hydrolysis in case of *B. aroideae*, and *B. carotovorum* were negative. When fructose was added to inulin in the culture media inoculated with *B. aroideae*, and *B. carotovorum* it induced complete stoppage of inulin utilization presumably on account of the availability of fructose on the one hand and the inhibitory action of this sugar on the β -h-fructosidase activity on the other. In case of *B. phytophthorum* the amounts of inulin utilised in 24 hours were not appreciably affected by the inclusion of fructose in the culture media, a result which would indicate that inulin utilization by this organism is, at least partly, mediated by a specific inulase enzyme. The observed reduction in the amount of inulin utilized may however, be due to the availability of fructose. Further the amount of inulin hydrolyzed by killed cells of *B. phytophthorum* was found to be more than the amount of sucrose hydrolyzed by similar cells, thus supporting the view that a specific inulase is present in cells of this organism, lest inulin should have been hydrolyzed at a much slower rate than sucrose if the process is taking place through the mediation of β -h-fructosidase.

Summary

The three closely related soft rot bacteria, *B. aroideae*, *B. carotovorum*, and *B. phytophthorum* hydrolyze starch before utilization by a constitutive amylase enzyme, probably an α -amylase, located at the protoplasmic surfaces of the bacterial cells. Inulin utilization is accomplished by a β -h-fructosidase enzyme in case of *B. aroideae*, and *B. carotovorum* and is, at least partly, mediated by a specific inulase in case of *B. phytophthorum*.

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